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Characterization of a new component and impurities in josamycin by trap-free two-dimensional liquid chromatography coupled to ion trap time-of-flight mass spectrometry

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Rationale: Impurities' toxicities of drug will affect the clinical effect and cause potential health risk, therefore, it is essential to study impurities profiles. In this study a new structural type of component and two acid degradation impurities in josamycin were discovered and characterized for the further improvement of official monographs in pharmacopoeias.
Methods: The component and acid degradation impurities in josamycin were separated and preliminary characterized by a trap-free two-dimensional liquid chromatography coupled to high resolution ion trap time-of-flight mass spectrometry (2D LC/IT-TOF MS) with both positive and negative electrospray ionization modes. Eluent of each peak from the first dimensional chromatographic system was trapped by a switching valve and subsequently transferred to the second dimensional chromatographic system, which was connected to MS. Full scan MS was firstly conducted to obtain the exact *m/z* values of the molecules. Then LC-MS/MS and LC MS/MS/MS experiments were performed on the compounds of interest.
Results: A new structural type of component, which was named as josamycin A, and two acid degradation impuritiess, which were identified as impurity Ⅰ and impurity Ⅱ, were discovered in josamycin. Their structures and fragmentation pattern were deduced according to MS^n data. Furthermore, the josamycin A was synthesized and impurity Ⅰ was separated by preparative HPLC. The structures of josamycin A and impurities were confirmed by ^1H NMR and ^{13}C NMR data.
Conclusions: Formation mechanism: Josamycin A was produced when the hydroxyl group on the macrolide of josamycin was oxidized into carbonyl. Impurity Ⅰ and impurity Ⅱ were produced by the loss of one molecule of acetyl mycaminose from josamycin and josamycin A, respectively. Compared with josamycin, the experiment results showed that josamycin A had a higher antibacterial activity with similar cytotoxicity, while impurity Ⅰ had no antibacterial activity but a higher cytotoxicity. As a result, the control of impurity Ⅰ is significant.
Keywords: josamycin, new component, impurity, trap-free 2D LC/IT-TOF MS

1 INTRODUCTION

Many 16-membered macrolides and their derivatives had relatively good gastric tolerance and low tendency to produce allergy and induce resistance[1]. Josamycin and its derivatives are typical examples[2-6]. Josamycin (leucomycin A_3) produced by fermentation of *streptomyces narbonensis var.* Josamyceticus[7], is consist of 16-membered ring aglycone and disaccharide (Figure 1).Similar to erythromycin[8], josamycin exhibited a broad-spectrum antibacterial activities, and was used in the clinical treatment of respiratory tract infections and sexually transmitted diseases, caused by Gram-positive bacteria, which includes mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, and bordetella pertussis[9-10]. In particular, josamycin is an alternative medicine for patients exhibiting penicillin allergy and developing resistances to penicillin[11-12] and 14- and 15-membered macrolides[13]. The immunomodulatory properties independent of its antibacterial actions of josamycin may have clinical relevance to inflammation, such as periodontal diseases[14]. In addition, the current reports on synthesized leucomycin analogues have revealed high antibacterial activity and anticancer activity, which indicated that these types of antibiotics can be good starting material for antibacterial and anticancer drug candidates[15].

However, the components and impurities of macrolide antibiotics are generally complicated because commercial josamycin is produced through fermentation and extracted from a mixture of numerous structurally related compounds. The compounds possessing antibacterial activities were called as components, and had potential to develop into new drugs. The compounds which have no antibacterial activity but toxicity were called as impurities, and their control is critical during the manufacturing of drugs. Guidelines on the identification and qualification of impurities above a certain threshold have been issued by the International Conference on Harmonization of Requirements for Registration Pharmaceuticals for Human Use (ICH)[16]. Therefore, the structures of these components and impurities should be characterized to ensure the effectivity and safety.

The two-dimensional liquid chromatography coupled to high resolution ion trap/time-of-flight mass spectrometry (2D LC/ IT-TOF MS) had great advantage in establishments of impurity profiles[17-19]. The 2D LC/MS overcomes the drawbacks of incompatibility between non-volatile salt mobile phase and ionization process in mass spectrometry. The TOF MS is capable to characterize minor complex structural components at trace levels due to its high sensitivity, efficiency and selectivity[20-22]. In this article, trap-free 2D LC coupled to IT-TOF MS has applied to be separated and identified the components and impurities in josamycin. Each peak eluted from the first dimensional chromatography was trapped by a switching valve and sent to the second dimensional chromatography for desalting. This 2D chromatography successfully convert the non-volatile mobile phase in the first dimension to volatile mobile phase in the second dimension before entering IT-TOF MS for analysis.

The known component and impurities in josamycin were determined by HPLC at detection wavelength of 231 nm according to Chinese Pharmacopoeia. However, a new structural type of component and acid degradation impurities in josamycin were discovered at a detection wavelength of 280 nm. Although some researchers have characterized some impurities in josamycin using LC/MS approach[23-27], the components and acid degradation impurities of josamycin discovered in this study have not been reported. As presented in

Figure 1, the structures of a new component which was named as josamycin A, and two acid degradation impurities in josamycin were characterized preliminarily by 2D LC/IT-TOF MS with positive and negative electrospray ionization modes. Based on MS^n data, the structure and fragmentation pathways of new component and acid degradation impurities of josamycin were elucidated by referring to published article on structural analogues[28]. Subsequently, josamycin A was synthesized and degradation impurity was separated by preparative HPLC for structural confirmation, and the structures of josamycin A and impurities were confirmed by NMR. The forming mechanisms of josamycin A and degradation impurities in josamycin were also studied. Furthermore, antibacterial activity and cytotoxicity of these component and acid degradation impurities in josamycin were studied.

2 EXPERIMENTAL

2.1 Materials

Josamycin (batch number: 13359-201302) used in this study was purchased from National Institutes for Food and Drug Control (Beijing, China). HPLC grade methanol was purchased from Merck (Darmstadt, Germany), ammonium acetate and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, USA). Analytical grade hydrochloric acid was purchased from Lanxi Yongli Chemical Industry Co. Ltd. (Jinhua, China), Analytical grade Sodium hydroxide was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and de-ionized water used in this study was producedby a Millipore Milli-Q-Gradient purification system.

2.2 Trap-free two-dimensional LC apparatus

A trap-free two-dimensional Nexera-XR liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with four binary pumps was used. The first dimensional liquid chromatography system included two binary pumps (LC-30AD), an auto-sampler (SIL-30AC), a column thermostat (CTO-20A) and a diode array detector (SPD-M20A). Chromatographic separation in the first dimension was carried out at 60℃ using a ZORBAX SB-C18 analytical column (150 mm × 4.6 mm, 3.5μm). The mobile phase of the first dimension were (A) 0.1 M ammonium acetate solution, whose pH was adjusted to 4.5 with phosphoric acid, and (B) methanol-acetonitrile (55:5, v/v). The elution program were as follows: 0 min, 40% B (hold for 10 min); 30 min, 60% B; 45 min, 60% B; 46 min 40% B (hold for 9 min). The mobile phase flow rate was 1.0 mL min^{-1} and injection volume was 10 μL. The second dimensional chromatography system was consisted of two binary pumps (LC-20AD), a column thermostat (CTO-20A) and a photo diode array detector (SPD-20A). Chromatographic separation in the second dimension was carried out at 60 ℃ using a Shimadzu Shim-pack GISS C_{18} analytical column (50 mm × 2.1 mm, 1.9 μm). The mobile phase of the second dimension were (A) 20 mM ammonium acetate solution and (B) methanol with gradient conditions: 0 min, 5% B; 5 min, 95% B; 5.5 min, 5% B; 9 min, 5% B. The mobile phase flow rate was 0.3 mL min^{-1}. And the detection wavelengths of UV detector were set at 231 nm and 280 nm simultaneously. The first and second dimensional columns systems were connected by means of two high pressure six-position and six-port switching valves, which were equipped with six 20 μL stainless steel quantitative loops. Loop 1~5 were used to retain target component, loop 6 was used as a drain loop to exchange the first and

second dimension mobile phase The first dimension column effluent was detected by diode array detector, and then target peak was fractioned in sample loops based on their retention time. The first dimension column effluent was sent to sample loop just before the target peak appeared, the flow line was switched to waste when the target peak reached the loop center. The second dimension mobile phase directly flowed to the MS (or drain) without passing through any loops in the initial state. During LC-MS analysis, the second dimension mobile phase flowed and pushed the sample within loop to second dimension column, then to MS.

2.3 MS spectrometry

Trap-free two-dimensional liquid chromatography coupled to 2D LC/IT-TOF MS from Shimadzu Corp. (Kyoto, Japan). The mass spectrometry detector (MSD) was equipped with an electrospray ionization (ESI) source. The ionization mode was positive and negative alternatingly. The interface and MS parameters were as the follows: nebulizer gas flow: 1.5 L min^{-1} (nitrogen); Collision gas: argon; the temperature of curve desolvation line (CDL) and heat block: 200 ℃; detector voltage: 1.56 V; scan range: 100-1000 *m/z*; the mode of MS^n: manual to MS^n. The mode of ESI was in both positive and negative ion mode.

2.4 Software

All data acquired were processed by Shimadzu LC-solution software (Kyoto, Japan). Formula predictor from Shimadzu Corp. (Kyoto, Japan) was used to simulate and study the fragmentation behavior of the described compounds.

2.5 NMR spectroscopy

All NMR spectra measurements were performed on a Bruker AVIII 600MHz spectrometer (^{1}H=600 MHz, ^{13}C=101 or 151MHz) at 25℃ using $CDCl_3$ as the solvent. ^{1}H NMR and ^{13}C NMR chemical shift values were reported on the δ scale in ppm, relative to trimethylsilane (TMS) (=0.00).

2.6 Sample preparation

Preparation of josamycin solution: The bulk drug of josamycin was dissolved in ammonium acetate solution (0.1 M, pH 4.5)-methanol-acetonitrile (40:55:5, v/v) solution and the concentration was 2.0 mg ml^{-1}.

Acid degradation: 50 mg of josamycin was dissolved by 1 mL hydrochloric acid at the concentration of 0.1 M and kept for 2 hours in water bath at the temperature of 37℃. After 2 hours, the solution was neutralized by 0.1 M sodium hydroxide and diluted with ammonium acetate solution (0.1 M, pH 4.5)-methanol-acetonitrile (40:55:5, v/v) to 10 mL.

3 RESULTS AND DISCUSSION

3.1 Structural elucidation of josamycin A

The first dimensional chromatogram of josamycin were shown in Figure 2. Figure 2A showed chromatogram of josamycin detected at the wavelength of 231 nm, and Figure 2B showed chromatogram of josamycin detected at the wavelength of 280 nm. Josamycin A was discovered and detected at the wavelength of 280 nm. ESI-MS exact mass data and theoretical mass data on $[M+H]^+$ and $[M-H]^-$ of the new component in josamycin were shown

in Table 1, the values of deviation in both positive ion and negative ion modes were basically less than 5 ppm. ESI-MS^n exact mass data on major product ions of the new component in josamycin in positive ion modes were shown in Table 2.

The formula of josamycin A was $C_{42}H_{67}NO_{15}$ based on the data obtained from TOF MS as shown in Figure 3. Figure 4 presented the proposed structure and fragmentation pattern of josamycin A. Josamycin A exhibited $[M+H]^+$ at *m/z* 826.4592 and $[M-H]^-$ at *m/z* 824.4439 indicated that the molecular weight of josamycin A was 825, which was 2 Da less than that of josamycin. The protonated molecule at *m/z* 826.4592 of josamycin A fragmented into the product ion at *m/z* 598.3113 by a loss of 228 Da, which corresponded to the glycosidic cleavage between the mycaminose and the mycarose. In the MS^3 spectrum of the fragment ion at *m/z* 598.3113 resulted in the ion at *m/z* 407.2010 and *m/z* 192.1227, which corresponded to the cleavage of the ether bond between 16- membered ring and mycaminose. The fragmentation pathways of losingone mycarose and one mycaminose was in agreement with the published paper[28]. Furthermore, the fragment ion at *m/z* 407.2010 lost one H_2O, one CH_3COOH and one CH_3OH successively resulted in the product ion at *m/z* 389.1929, *m/z* 329.1714 and *m/z* 297.1460, respectively. Another fragmentation pathway of the product ion at *m/z* 407.2010 was losing one CH_3COOH, one CH_3OH and one H_2O successively and resulted in the ion at *m/z* 347.1788, *m/z* 315.1564 and *m/z* 297.1460. The fragment ion at *m/z* 297.1460 further neutral loss of one CO resulted in the ion at *m/z* 269.1526. Besides, the ultraviolet absorption wavelength of the josamycin A shifted toward longer wavelength compared with josamycin, which indicated the increased conjugated structure. From the above information, josamycin A was an oxidation product of josamycin by oxidizing the hydroxyl group on the macrolide to carbonyl.

3.2 Synthesis of josamycin A

100 mL dichloromethane solution containing 1.2 g Dess-Martin periodinane was added to a 100 mL dichloromethane solution with 1.0 g josamycin as solute constant stirring were maintained at room temperature until the disappearance of josamycin, which was monitored by thin-layer chromatography. Solvent was removed under reduced pressure. The residue was purified by column chromatography to give the product as white powder. The synthetic route of josamycin A was shown in Figure 5.

3.3 Structural confirmation of synthetic josamycin A by ^1H NMR and ^{13}C NMR

All NMR spectra measurements were performed on a Bruker AVIII 600MHz spectrometer (^1H=600 MHz, ^{13}C=101 MHz) at 25℃ using $CDCl_3$ as the solvent. The ^1H NMR and ^{13}C NMR spectra of josamycin A and the correspondence between structural units and peaks in their NMR spectra were analyzed, the molecular structure of josamycin A together with the atom numbering and NMR data were shown in Figure 1 and Table S1. The changes of josamycin A in ^{13}C NMR chemical shift compared with josamycin and C-9 hydroxyl substituted josamycin derivatives[3-6] were clear. The presence of carbonyl group on position C-9 in 16-membered ring was proved by the analysis of the ^{13}C NMR spectra of josamycin A in which an additional signal at 202.8 ppm(C=O) was detected. The proton hydrogen signal on position C-9 disappeared, while proton and carbon signals assigned to the mycarose and mycaminose parts in josamycin A were not changed.

The data from the mass spectrum and LC retention time of josamycin A agree with the data obtained from synthetic josamycin A, which confirmed that josamycin A corresponded the structure of josamycin A.

3.4 Structural elucidation of acid degradation impurities

Figure 6 showed the first dimensional chromatogram of josamycin after acid degradation, impurity Ⅰ was detected in josamycin after acid degradation at the wavelength of 231nm as presented in figure 6A and impurity Ⅱ at 280 nm as presented in figure 6B. The ESI-MS exact mass data and theoretical mass data on $[M+H]^+$ and $[M-H]^-$ of the two acid degradation impurities in josamycin in both positive and negative ionization modes were shown in Table 1 and the values of deviation were basically less than 5 ppm. ESI-MS^n exact mass data of major product ions of the impurities in josamycin in positive ionization mode were shown in Table 2.

The formula of impurity Ⅰ was $C_{30}H_{49}NO_{11}$ based on the data from TOF high resolution mass spectrometer. Figure 7 presented the proposed structure and fragmentation pattern of impurity Ⅰ. The fragmentation pathway of the protonated molecule at *m/z* 600.3400 concerned the loss of one H_2O and led to the ion at *m/z* 582.3244. Subsequently, the cleavage of ether bond occurred and the loss of $C_8H_{17}NO_4$ from the ion at *m/z* 582.3244, which produced the ion at *m/z* 391.2130. Further loss of one CH_3COOH and one CH_3OH from the ion at *m/z* 391.2130 conducted to the ions at *m/z* 331.1892 and *m/z* 299.1646, respectively. The fragmentation pathway of the product ion at *m/z* 299.1646 concerned the loss of 18 Da, which was identified as H_2O, and led to the ion at *m/z* 281.1532. From the above information, it was concluded that the impurity Ⅰ was an acid degradation impurity.

The formula of impurity Ⅱ was $C_{30}H_{47}NO_{11}$ based on the data from TOF high resolution mass spectrometer. Impurity Ⅱ exhibited $[M+H]^+$ at *m/z* 598.3239 and $[M-H]^-$ at *m/z* 596.3094, which indicated that the molecular weight of impurity Ⅱ was 597 and 2 Da less than impurity Ⅰ. Figure 8 presented the proposed structure and fragmentation pattern of Impurity Ⅱ. The deprotonated molecule at *m/z* 596.3094 initially gave rise to the ion at *m/z* 536.2922 due to the loss of one CH_3COOH. Moreover, the ultraviolet absorption wavelength of impurity Ⅱ shifted toward longer wavelength compared with that of impurity Ⅰ. Therefore, the structure of impurity Ⅱ was that the hydroxyl group on the macrolide of impurity Ⅰ was oxidized to carbonyl, resulting in impurity Ⅱ conjugated chains lengthened and absorbing wavelength redshift.

3.5 Preparation of impurity Ⅰ

1.0 g of josamycin was dissolved by 100 mL 0.1 M hydrochloric acid and the solution was kept for 2 hours in water bath at 37℃. After 2 hours, the solution was neutralized by 0.1 M sodium hydroxide, which was furtherly extracted with dichloromethane three times with 50 mL each time. The extracted dichloromethane solution was combined organic layer and dried using anhydrous Na_2SO_4. The white powder was obtained by evaporating solvent under reduced pressure. Then, the impurity Ⅰ was isolated using a Shimadzu semi-preparative HPLC.

3.6 Structural confirmation of impurity Ⅰ by ^{1}H NMR and ^{13}C NMR

All NMR spectra measurements were performed on a Bruker AVIII 600MHz spectrometer (^{1}H=600 MHz, ^{13}C=151 MHz) at 25℃using $CDCl_3$ as the solvent. The ^{1}H NMR and ^{13}C NMR spectra of impurity Ⅰ and correspondence between structural units and peaks in their NMR spectra were analyzed. The molecular structure of impurity Ⅰ together with the atom numbering and NMR data were shown in Figure 1 and Table S2. Comparison of the NMR data between josamycin[5] and impurity Ⅰ showed that signals assigned 16-membered ring and mycaminose were similar. The proton and carbon signals assigned to the mycarose were not detected in impurity Ⅰ, which means the loss of mycarose

3.7 Forming mechanism, antibacterial activity and cytotoxicity of josamycin A and degradation impurities in josamycin

Josamycin A was an oxidative product of josamycin as the hydroxyl group on the macrolide was oxidized to carbonyl. Impurity Ⅰ was produced by the loss of one molecule of acetyl mycaminose from josamycin, and impurity Ⅱ produced by the loss of one molecule of mycaminose from josamycin A under acidic condition. The Impurity Ⅰ and impurity Ⅱ were existed and could be detected in commercial josamycin. Because of the increased conjugated structure, the ultraviolet absorption wavelengths of the josamycin A and impurity Ⅱ shifted toward longer wavelength compared with josamycin and impurity Ⅰ. Josamycin and impurity Ⅰ exhibited the maximum absorption at the wavelength of 231 nm, whereas josamycin A and impurity Ⅱ showed the maximum absorption at the wavelength of 280 nm. The formation mechanisms of josamycin A and acid degradation impurities were shown in Figure 1.

The antibacterial activity was determined by cup-plate method (two-dose assay) reference to the statistical method of biological assay in the Chinese Pharmacopoeia 2015. The result showed that the activity of josamycin A was 1.5 times high josamycin and the impurity Ⅰ had no antibacterial activity. Whereas, the experimental result of cytotoxicity showed that there was no significant difference between the josamycin A and josamycin, and the survival ratio of cell L929 was identical under the five concentrations of josamycin A and josamycin. Impurity Ⅰ showed higher cytotoxicity than josamycin, and the survival ratio of cell L929 was lower with the application of impurity Ⅰ. The relationship of drug concentration and bacteriostatic rate was shown in Figure 9. This result indicated that content of impurity Ⅰ should be control in josamycin.

4 CONCLUSIONS

The structures of a new component and two acid degradation impurities in josamycin were discovered and identified by 2D LC/IT-TOF MS. Josamycin A (new component) was synthesized and impurity Ⅰ was separated by preparative HPLC, and their structures was confirmed by ^{1}H NMR and ^{13}C NMR data. Josamycin A was an oxidative product of josamycin, whose the hydroxyl group on the macrolide were turned into carbonyl during the oxidation process. Impurity Ⅰ was produced by the loss of one molecule of acetyl mycaminose from josamycin, and impurity Ⅱ was produced when josamycin A lost one molecule of mycaminose. Compared with josamycin, experiment results showed that josamycin A presented a higher antibacterial activity and little difference in cytotoxicity, and impurity Ⅰ had no antibacterial activity but a higher cytotoxicity. Therefore, impurity Ⅰ

should be controlled. Based on characterization of impurities, this study revealed the mechanism of impurity production and can provide guidance for pharmaceutical companies to improve the manufacturing process and reduce impurity content. The result from this research also established scientific basis for quality control of josamycin.

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TABLE 1 ESI-MS exact mass data and theoretical mass data on $[M+H]^+$ and $[M-H]^-$ of josamycin A and impurities in josamycin in positive ion (a) and negative ion (b) modes.

Comp.	$[M+H]^+$ (m/z)	formula	Theoretical $[M+H^+]$(m/z)	Deviation[b] (ppm)	$[M-H]^-$ (m/z)	formula	Theoretical $[M-H]^-$(m/z)	Deviation[b] (ppm)
A	826.4592	$C_{42}H_{68}NO_{15}$	826.4582	1.21	824.4439	$C_{42}H_{66}NO_{15}$	824.4438	0.12
I	600.3389	$C_{30}H_{50}NO_{11}$	600.3378	1.83	598.3217	$C_{30}H_{48}NO_{11}$	598.3233	-2.67
II	598.3204	$C_{30}H_{48}NO_{11}$	598.3222	-3.01	596.3094	$C_{30}H_{46}NO_{11}$	596.3094	3.02

a: the data of theoretical $[M+H]^+$ and $[M-H]^-$ was calculated by software of Shimadzu ccurate Mass calculator.

b: the deviation was calculated by the following formula: $(M-M_0)/m*10^6$, where M was the measured value of the ion mass; M_0 was the theoretical value of the ion mass; m was the mass of the ion.

TABLE 2 ESI-MS^n exact mass data on major product ions of the josamycin A and impurities in joamycin in positive ion (a) and negative ion (b) modes.

(a) positive				
Name	$[M+H]^+$ (*m/z*)	MS/MS fragmentation ions (*m/z*)	Precursor ion (*m/z*) in MS/MS/MS	MS/MS/MS fragmentation ions (*m/z*)
Josamycin A	826.4592	598.3113，407.2010	598.31	407.2007，389.1929，375.2253，347.1788，329.1714，315.1564，297.1460
Impurity I	600.3389	582.3203, 391.2114, 331.1930, 299.1653, 281.1541, 192.1267	582.32	391.2148
(b) negative				
Name	$[M-H]^-$ (*m/z*)	MS/MS fragmentation ions (*m/z*)	Precursor ion (*m/z*) in MS/MS/MS	MS/MS/MS fragmentation ions (*m/z*)
Impurity II	596.3094	536.2922		

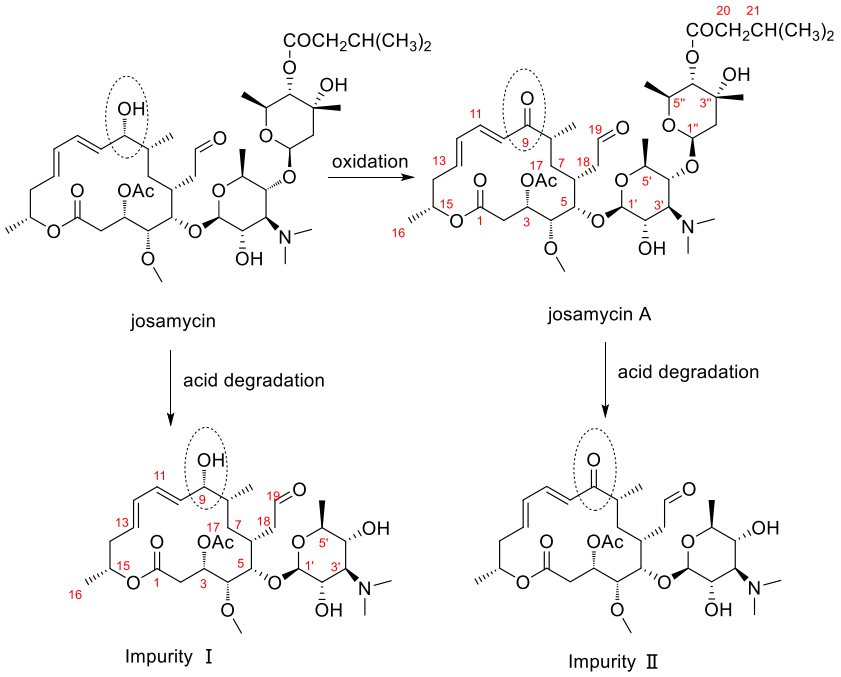

FIGURE 1 The forming mechanisms and chemical structures of josamycin A and acid degradation impurities. The dotted lines marked their main differences in 16-membered ring.

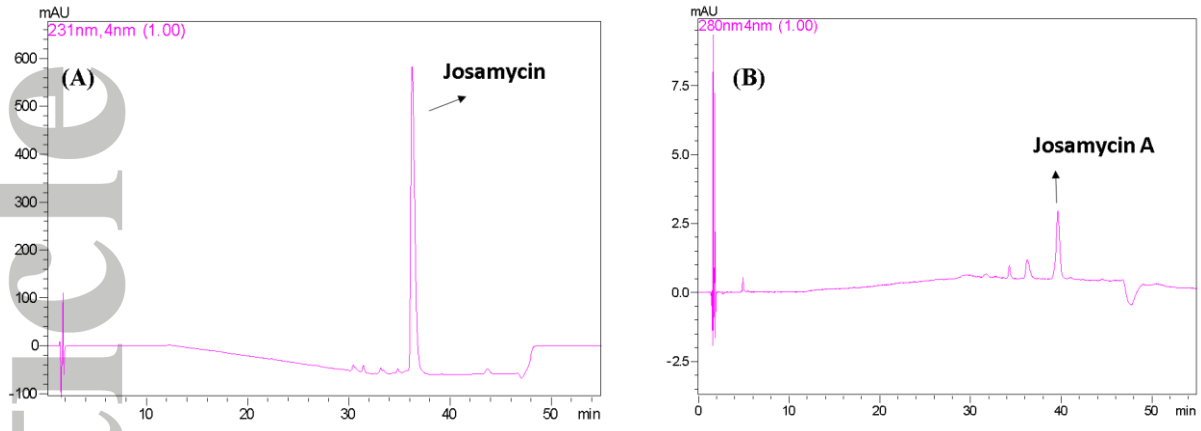

FIGURE 2 The first dimensional chromatogram of josamycin, (A) detected at the wavelength of 231 nm, (B) detected at the wavelength of 280 nm.

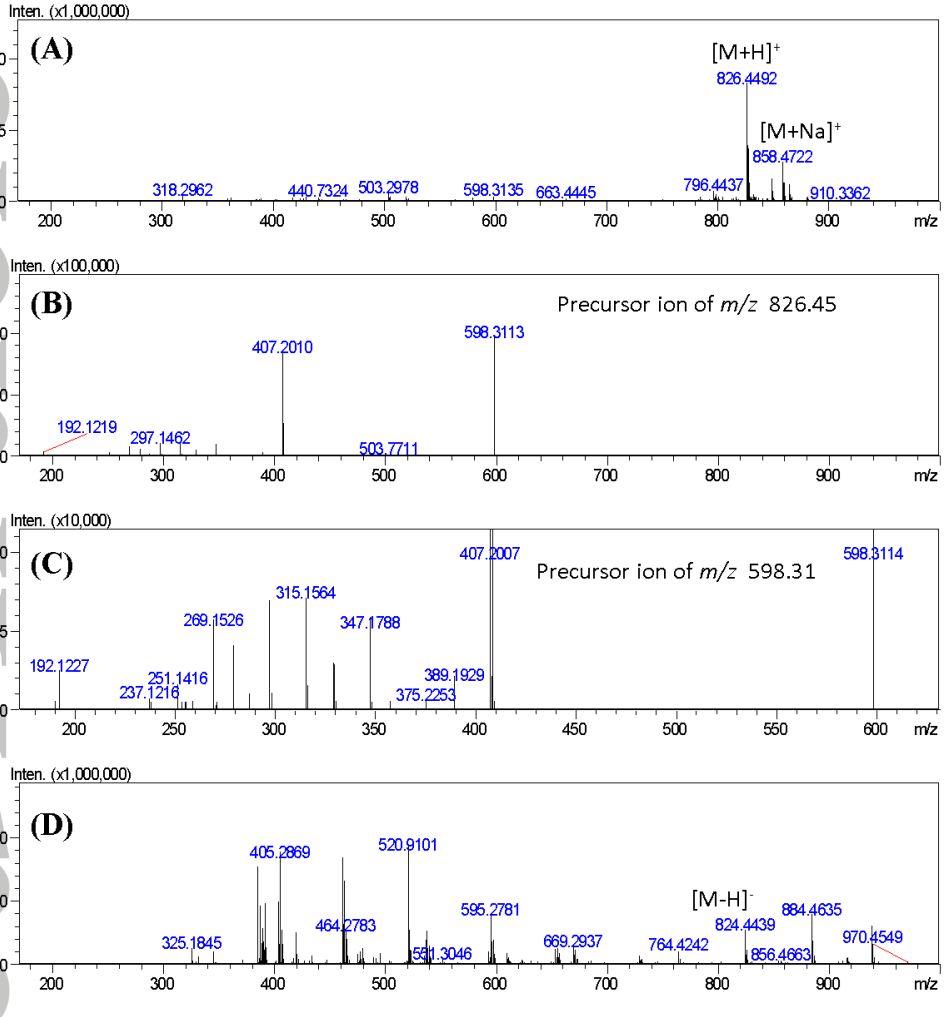

FIGURE 3 MS full scan ESI^+ (A) and ESI^- (D), the MS^2 (B) and MS^3 (C) spectra in both positive ionization of josamycin A.

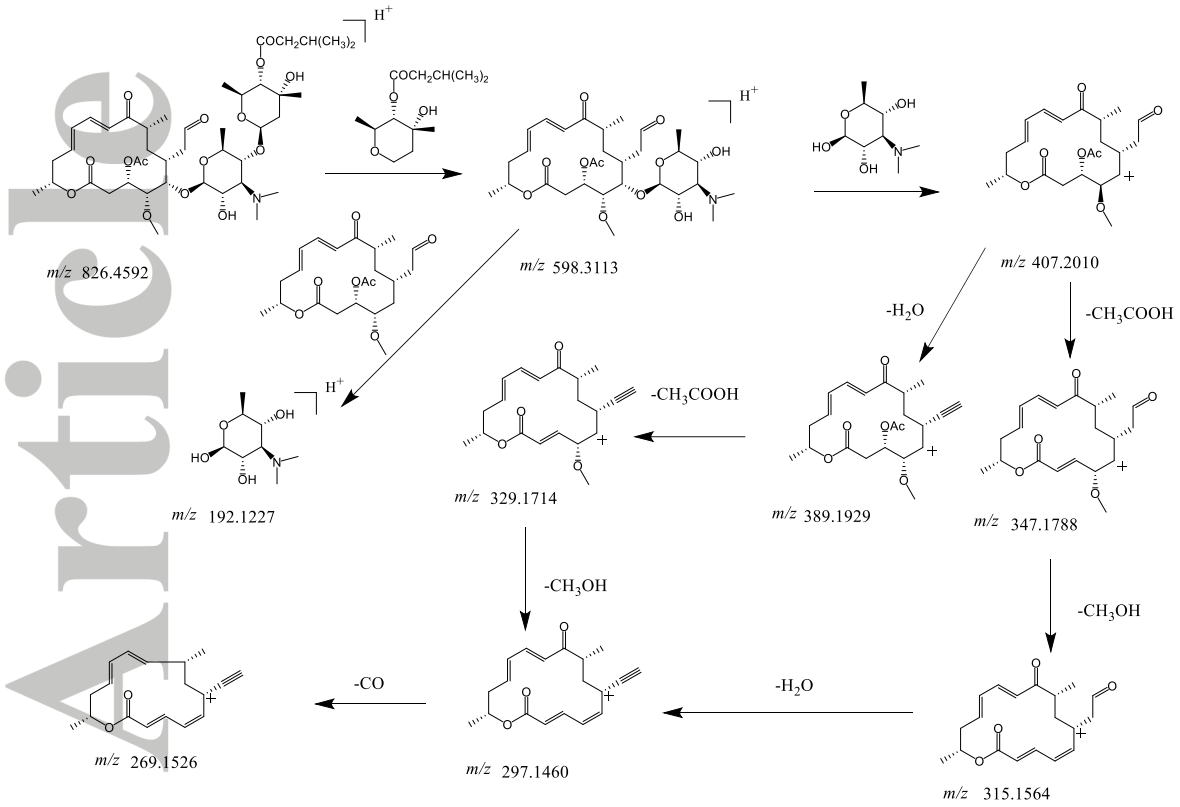

FIGURE 4 Proposed fragmentation pathways of josamycin A in the positive ion mode.

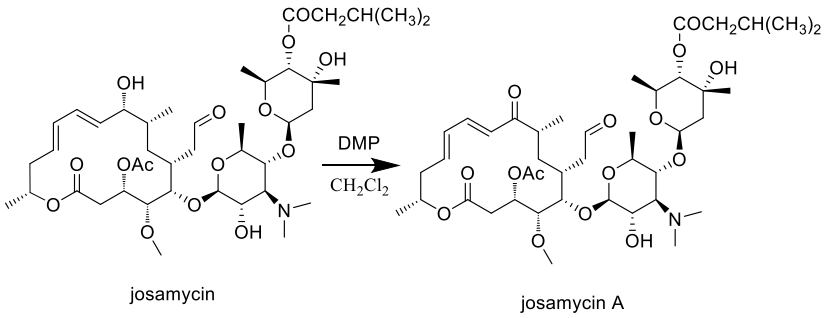

FIGURE 5 Structure and synthetic route of josamycin A.

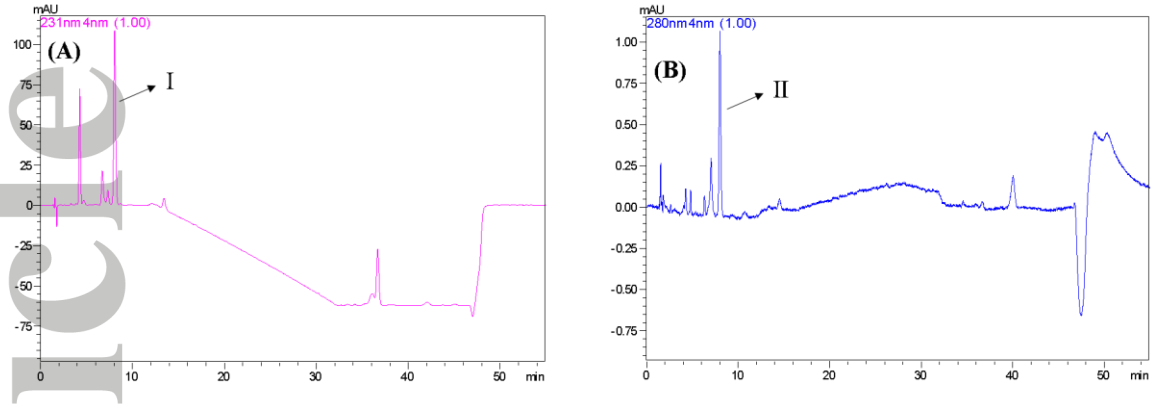

FIGURE 6 First dimensional chromatogram of josamycin after acid degradation, (A) detected at the wavelength of 231 nm, (B) detected at the wavelength of 280 nm.

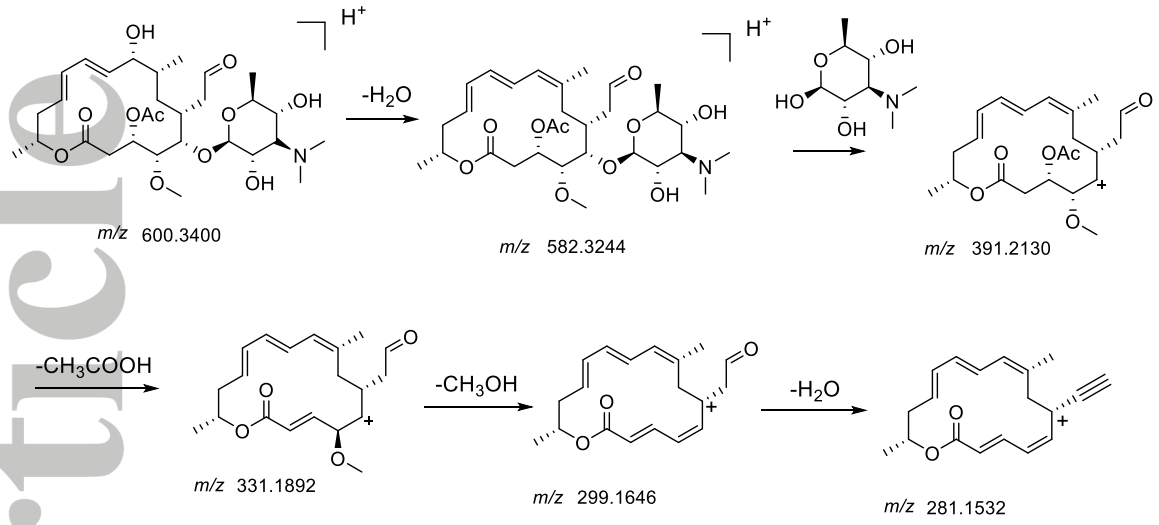

FIGURE 7 Proposed fragmentation pathways of impurity Ⅰ in the positive ion mode.

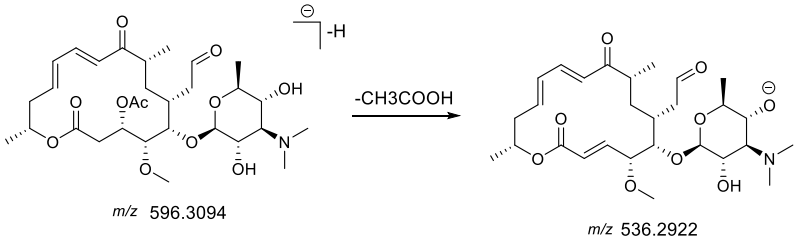

FIGURE 8 Proposed fragmentation pathways of impurity Ⅱ in the negative ion mode.

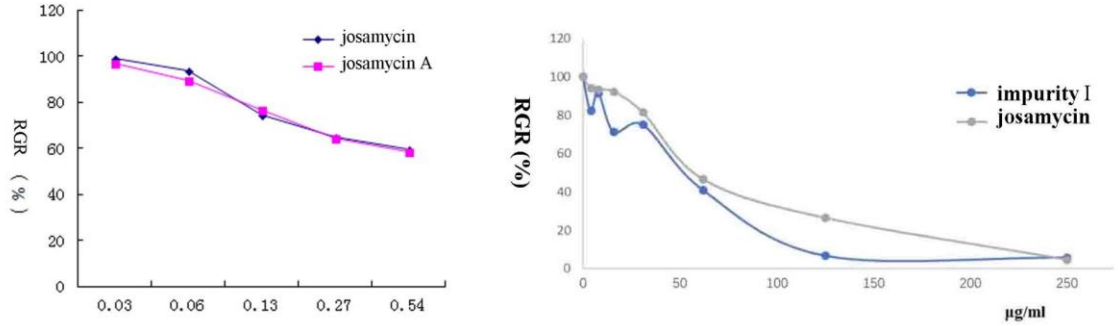

FIGURE 9 Relationship of drug concentration and bacteriostatic rate by MTT assay. The absorbance was measured at 490 nm, relative growth ratio (RGR) was calculated according to following formula: RGR% =Average absorbance of test group/ Average absorbance of control group×100%.