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Acid catalysed degradation of some spiramycin derivatives found in the antibiotic bitespiramycin

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Abstract

Bitespiramycin is a novel antibiotic containing a number of 4″-acylated spiramycin derivatives (isovalerylspiramycins I–III, butanoylspiramycin III, propanoylspiramycin III and acetylspiramycin III) as major components. These spiramycin derivatives are susceptible to degradation in acid solution. Liquid chromatography–ion trap mass spectrometry (LC/MS^n) was used to study the degradation of these spiramycin derivatives in simulated gastric fluid at 37 °C. All derivatives degraded by first-order reactions for which rate constants (k) and half-lives ($t_{1/2}$) were calculated. Acyl groups at position 3 had less effect on acid-stability of spiramycin derivatives than acyl groups at position 4″. The introduction of 4″-acyl groups enhanced the acid-stability of spiramycin derivatives and altered the degradation pathway in simulated gastric fluid such that loss of forosamine rather than loss of mycarose becomes the major degradation pathway.

Keywords: Bitespiramycin; Spiramycin; Degradation; Liquid chromatography–ion trap mass spectrometry

1. Introduction

Spiramycin is a 16-member macrolide antibiotic used to treat infections of the oropharynx, respiratory system and genito-urinary tract as well as cryptosporidiosis and toxoplasmosis [1–3]. The drug is actually a mixture of spiramycin I together with its 3-acetyl (spiramycin II) and 3-propanoyl (spiramycin III) esters (Table 1). The relative amounts of these three components vary in different countries. For instance in France the product contains mainly spiramycin I (over 85%) with smaller amounts of spiramycins II and III (<5 and 10%, respectively) [4] whereas in the Chinese product, spiramycin III is the major component and spiramycins I and II are minor components (<10 and 30%, respectively) [5].

Bitespiramycin is a novel antibiotic containing a group of 4″-acylated spiramycins particularly isovalerylspiramycins I (7.4%), II (22.5%) and III (37.7%). A number of other derivatives of spiramycin are also present as minor components including butanoylspiramycin III, propanoylspiramycin III, acetylspiramycin III and spiramycin III (Table 1). The drug is produced by recombinant *Streptomyces spiramyceticus* F21 [6] and is currently undergoing phase II clinical trials in China. Oral bitespiramycin has been shown to have improved bioavailability compared with spiramycin [7] and there are reports that spiramycin derivatives with longer carbon chains at positions 3 and 4″ have better antibiotic activity due to their higher lipophilicity [8–11]. However, the possibility that this higher bioavailability and antibiotic activity are the result of greater chemical stability in the stomach has not been investigated.

Spiramycin is unstable in acid due to loss of the mycarose residue [12,13]. The neospiramycin produced is inactive and undergoes further transformation to forocidin by loss of the forosamine residue [13]. The degradation products of 4″-acylated spiramycin derivatives have not been characterized. Spectrophotometry has been used to investigate the degradation kinetics of spiramycin [12], but the assay is not stability

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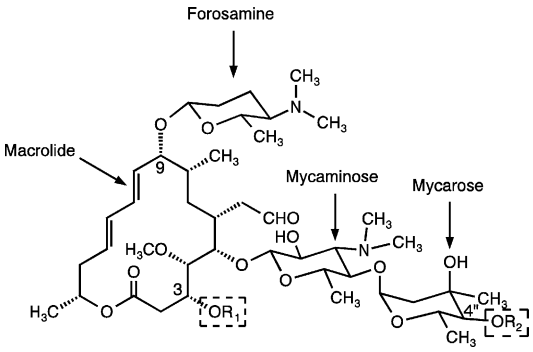
Table 1
Structures and mass spectrometry data of spiramycin derivatives investigated in simulated gastric fluid



	$[M + H]^+$	MS/MS	Components	R_1	R_2
A	927	768, 699, 540	Isovalerylspiramycin I	H	$COCH_2CH(CH_3)_2$
B	969	810, 741, 582	Isovalerylspiramycin II	$COCH_3$	$COCH_2CH(CH_3)_2$
C	983	824, 755, 596	Isovalerylspiramycin III	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$
D	969	810, 755, 596	Butanoylspiramycin III	$COCH_2CH_3$	$COCH_2CH_2CH_3$
E	955	796, 755, 596	Propanoylspiramycin III	$COCH_2CH_3$	$COCH_2CH_3$
F	941	782, 755, 596	Acetylspiramycin III	$COCH_2CH_3$	$COCH_3$
G	899	755, 740, 596	Spiramycin III	$COCH_2CH_3$	H
H	885	741, 726, 582	Spiramycin II	$COCH_3$	H
I	843	699, 684, 540	Spiramycin I	H	H

indicating. LC/MS^n substructural analysis methodologies have been developed to rapidly and accurately elucidate structures of degradants, which can serve to accelerate the drug discovery and development cycle [14]. The present work describes the application of an LC/MS^n method to identify the degradation products of spiramycin and the 4″-acylated spiramycin derivatives (Table 1) in simulated gastric fluid. The effect of acyl groups at positions 3 and 4″ on the kinetics of acid-catalysed degradation was also investigated using a quantitative method developed in our laboratory [15].

2. Materials and methods

2.1. Materials

Isovalerylspiramycins I–III, butanoylspiramycin III, propanoylspiramycin III, acetylspiramycin III, and platenomycin A1 were provided by the Institute of Medical Biotechnology (Beijing, China). Spiramycin (Chaoyang Pharmaceutical Factory, Liaoning, China) was separated by semipreparative HPLC to produce samples of spiramycins I–III. Neospiramycins I and III were produced by acid hydrolysis of spiramycin and isolated by semipreparative HPLC. Josamycin was purchased from Yamanouchi (Tokyo, Japan) and midecamycin A1 and pepsin from Sigma (St. Louis, USA). Leucomycin A1, roxithromycin and azithromycin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was HPLC grade and all other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10AD pump (Kyoto, Japan) connected to a Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm, Hi-Tech Scientific Instrument Co., Tianjin, China). Isocratic chromatography was performed using either mobile phase (A) of acetonitrile−10 mM ammonium acetate−acetic acid (35:65:0.5, v/v/v) for the separation of acetylspiramycin III and spiramycins I–III or mobile phase (B) of acetonitrile−10 mM ammonium acetate−acetic acid (45:55:0.5, v/v/v) for the separation of isovalerylspiramycins I–III, butanoylspiramycin III and propanoylspiramycin III. The flow rate was 0.5 ml/min and the column temperature was maintained at 25 °C.

A Finnigan LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA) was used for mass analysis and detection. The settings for the ESI ion source were: capillary temperature 170 °C, capillary voltage 6.0 V, and ion-spray-voltage 4.5 kV. Nitrogen was used as sheath gas (0.75 l/min) and auxiliary gas (0.15 l/min) and helium was used as collision gas (0.2 ml/min). The instrument was operated in the positive ion mode, producing $[M + H]^+$ and $[M + 2H]^{2+}$ ions by protonation of one or both tertiary amine groups. In qualitative experiments, singly charged ions were trapped and then fragmented by collision induced dissociation. Quantitation was performed using selected reaction monitoring (SRM) of doubly charged ions.

2.3. Preparation of solutions

Stock solutions of all spiramycin derivatives were prepared by dissolving reference substances in acetonitrile to give final concentrations of 400 μg/ml. The solutions were then successively diluted with acetonitrile to achieve standard solutions of 2000, 1600, 1000, 400, 200, 40 and 20 ng/ml for each analyte. Solutions with high, medium, and low concentrations of 1600, 400 and 40 ng/ml were used as quality control (QC) solutions. Internal standard solutions of azithromycin (1200 ng/ml) for use with mobile phase A and roxithromycin (800 ng/ml) for use with mobile phase B were also prepared in acetonitrile.

Simulated gastric fluid (mainly consisting of NaCl and HCl at pH 1.3) was prepared according to the USP XXV. The pH is similar to that of the medium used to study dissolution of acetylspiramycin tablets (pH 1.2) in the Chinese Pharmacopoeia (2000). All solutions were stored at 4 °C and brought to room temperature before use.

2.4. Degradation conditions

Stock solutions of spiramycin derivatives were diluted to a concentration of 200 μg/ml in acetonitrile. Simulated gastric fluid (6 ml) was added to each solution (0.2 ml) and incubated at 37 ± 1 °C for 2 h. An incubation of spiramycin derivatives was also carried out in the presence of pepsin. Samples (200 μl) were removed into glass tubes after 0.25, 0.5, 0.75, 1, 1.5 and 2 h and neutralized with 50 μl of 0.1 M Na_2CO_3 to terminate acid hydrolysis. Samples were then mixed with 50 μl of the appropriate internal standard solution and 100 μl of 0.1 M Na_2CO_3 (adjusted pH to 8.5), vortexed for 15 s and extracted with 2 ml of ethyl acetate–isopropanol (95:5, v/v) for 10 min on a roller-shaker. After centrifugation at 2000 × *g* for 10 min, organic layers were removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. Finally residues were dissolved in 100 μl aliquots of mobile phase, vortexed, and 20 μl injected into the LC/MS^n system. Every experiment was conducted in duplicate. Additional samples (200 μl) were removed at 1, 1.5 and 2 h, diluted with 500 μl acetonitrile, and directly injected into the LC/MS^n system in order to identify degradation products.

2.5. Data acquisition and analysis

Data were collected and analyzed using the ThermoFinnigan Xcalibur software package, Version 1.2. Peak integration and calibration were performed using Finnigan LCQuan software. Peak area ratios of analyte to internal standard were utilized for the construction of calibration curves. Degradation rate constants (*k*) were calculated by fitting the concentration–time data to pseudo-first-order kinetics.

2.6. Assay validation

Calibration curves for each analyte were prepared by analyzing calibration samples prepared by spiking blank simulated gastric fluid (200 μl) with standard solutions (50 μl) and adding 50 μl internal standard and 150 μl 0.1 M Na_2CO_3. QC samples were similarly prepared by spiking with QC solutions. Each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknown samples.

Linear calibration curves were obtained for each spiramycin derivative over the concentration range 20–2000 ng/ml in gastric fluid. The lower limit of quantitation (LOQ) was 20 ng/ml for each spiramycin derivative. Accuracy and precision were based on the results of QC samples (n = 18). Intra-run precision was assessed by one-way analysis of variance (ANOVA). Accuracy was expressed as the relative error (RE). The intra-run precision and accuracy for all analytes were less than 9.8% and ±5.2%, respectively.

3. Results and discussion

3.1. Identification of degradation products

Degradation of isovalerylspiramycins I–III, butanoylspiramycin III, propanoylspiramycin III, acetylspiramycin III and spiramycins I–III was shown to be independent of the presence of pepsin in the simulated gastric fluid.

3.1.1. Degradation of spiramycin III (G)

Degradation of spiramycin III produced two products G_1 and G_2. The full scan mass spectrum of G_1 had a pseudo-molecular ion at *m*/*z* 755 and a doubly charged ion at *m*/*z* 378 (Fig. 1A), indicating a molecular weight of 754 amu. The pseudo-molecular ion was 144 amu lower than that of spiramycin III, characteristic of the loss of the mycarose moiety. The doubly charged ion at *m*/*z* 378 indicates that the two tertiary amine groups are retained in the structure of G_1.

The MS/MS spectrum of G_1 shows a base peak at *m*/*z* 596 (Fig. 1B), suggesting the 16-member lactone nucleus is unaltered. The peak can be assigned to the product ion formed by loss of forosamine and a water molecule (−18 amu) from the precursor ion. This finding also confirms that the compound has retained the mycaminose moiety intact in the degradation process. To fully characterize the chemical structure of G_1, it was isolated by semipreparative HPLC and studied by NMR and MS/MS. In the ^{13}C NMR spectrum, signals due to mycarose were absent, whereas signals similar to those of spiramycin III were present. Thus, degradation product G_1 is confirmed to be neospiramycin III.

Degradation product G_2 gave a pseudo-molecular ion at *m*/*z* 614, indicating a molecular weight of 613 amu (Fig. 1C). This pseudo-molecular ion is 141 amu lower than that of neospiramycin III, characteristic of the further loss of the forosamine moiety. The compound generated major product ions at *m*/*z* 596, 423, and 405. Fragmentation ion *m*/*z* 596 was assigned as arising from loss of a water molecule and fragmentation ions *m*/*z* 423 and 405 were assigned as arising from loss of a molecule of mycaminose (−173 amu) followed

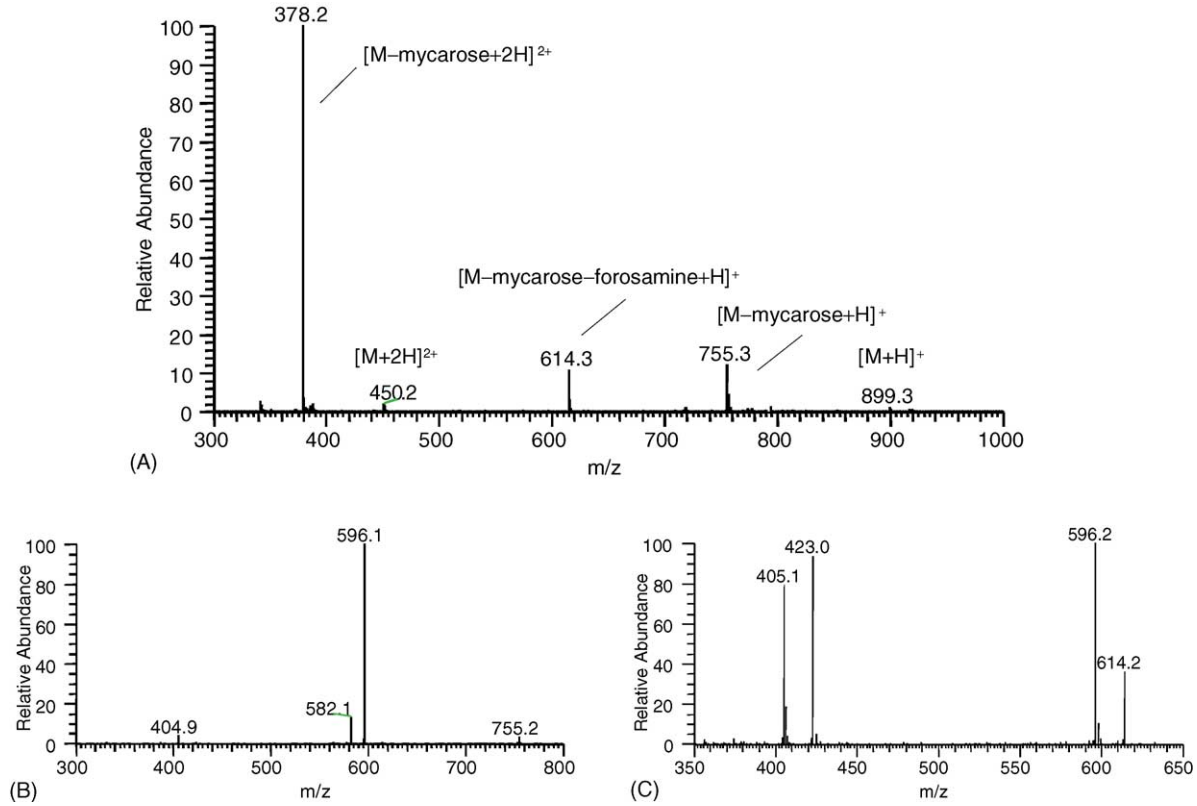


Fig. 1. Mass spectra of degradation products of spiramycin III (G, *m*/*z* 899) 1 h after incubation in simulated gastric fluid: (A) full scan mass spectrum; (B) MS/MS spectrum of *m*/*z* 755 (G_1); (C) MS/MS spectrum of *m*/*z* 614 (G_2).

by loss of a water molecule. Based on the reported degradation products of spiramycin, G_2 was assigned as forocidin III [13].

The proposed degradation pathway of spiramycin III in simulated gastric fluid is shown in Fig. 2. The degradation pathways of spiramycin I and II were similar to that of spiramycin III, consistent with previous results [12,13].

3.1.2. Degradation products of isovalerylspiramycin III (C)

Isovalerylspiramycin III gave rise to three degradation products C_1–C_3 in simulated gastric fluid. Like degradation product G_1, C_1 also gave a pseudo-molecular ion at *m*/*z* 755 (Fig. 3A). Based on the above data, C_1 was identified as neospiramycin III which was confirmed by comparison of its MS/MS spectrum with that of the reference substance.

Degradation product C_2 gave a pseudo-molecular ion at *m*/*z* 842 (Fig. 3A), whose intensity indicates it is the major product of degradation. It can be assigned as arising from loss of a molecule of forosamine from isovalerylspiramycin III. This assignment was supported by the fact that the doubly charged ion of C_2 was not observed in the full scan mass spectrum of degradation products of isovalerylspiramycin III. The MS/MS spectrum of C_2 showed a base peak at *m*/*z* 614 (Fig. 3B) assigned as arising from loss of a mycarose moiety from the precursor ion. C_2 was identified as platenomycin A1 which was confirmed by comparison of its MS/MS with that of a reference substance. Degradation product C_3 gave a

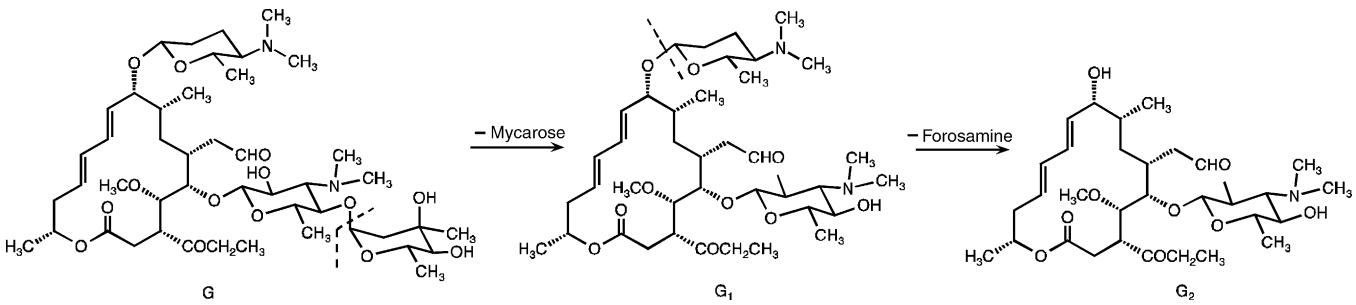


Fig. 2. Proposed degradation pathway of spiramycin III (G) in simulated gastric fluid.

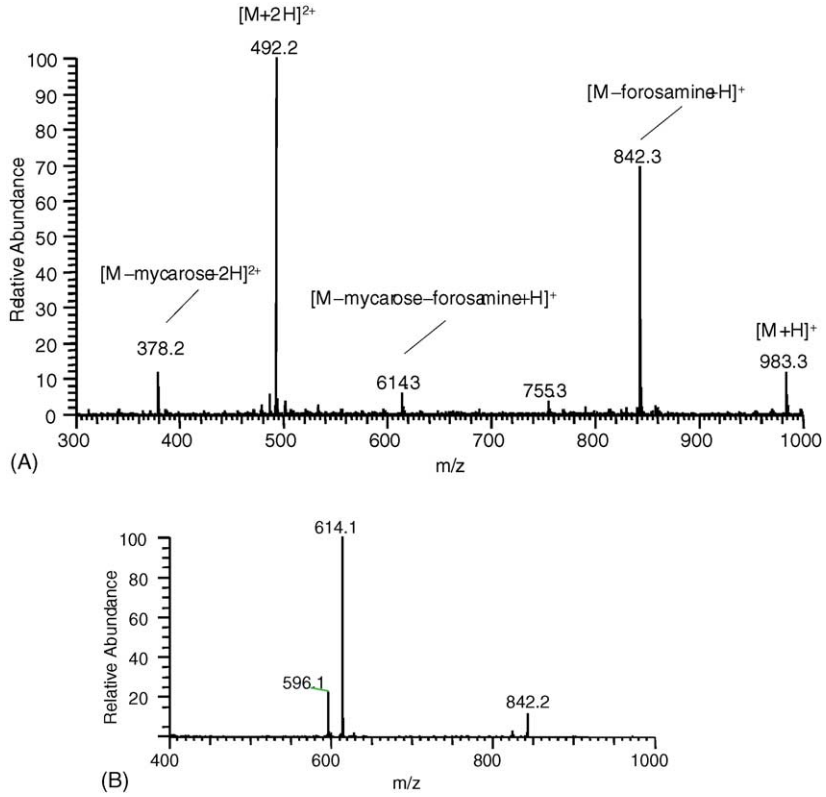


Fig. 3. Mass spectra of degradation products of isovalerylspiramycin III (C, *m*/*z* 983) 1 h after incubation in simulated gastric fluid: (A) full scan mass spectrum; (B) MS/MS spectrum of *m*/*z* 842 (C_2).

pseudo-molecular ion at *m*/*z* 614 and was assigned as forocidin III.

A proposed degradation pathway for isovalerylspiramycin III in simulated gastric fluid is shown in Fig. 4. The only difference in chemical structure between isovalerylspiramycin III and spiramycin III is at position 4″. However, loss of forosamine followed by loss of mycarose was the major decomposition pathway of isovalerylspiramycin III whereas loss of mycarose followed by loss of forosamine was the major decomposition pathway of spiramycin III (Fig. 2).

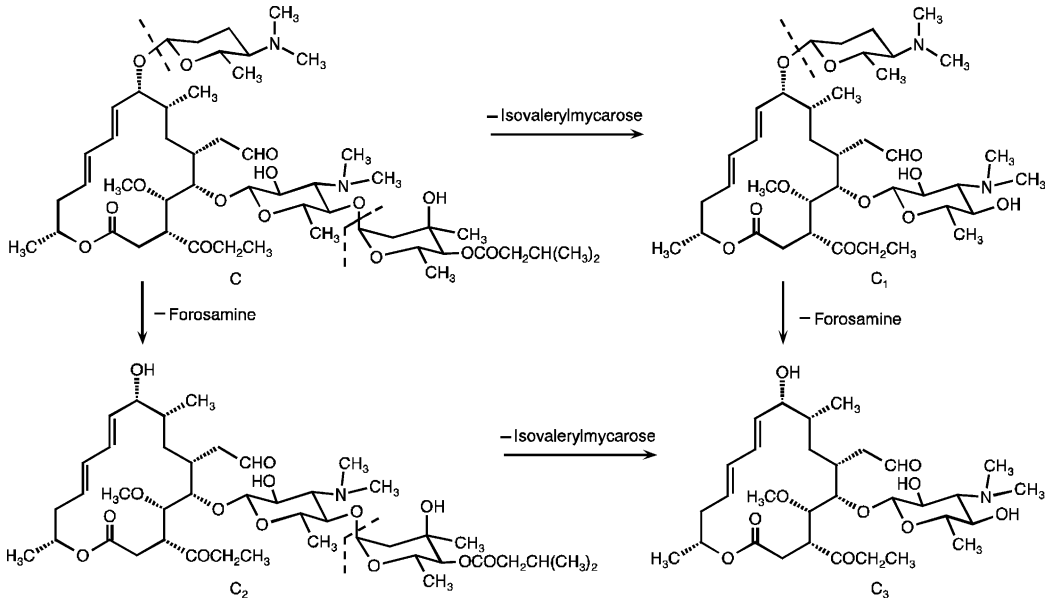
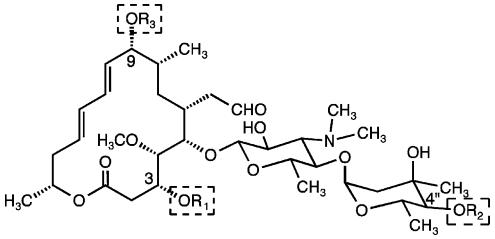


Fig. 4. Proposed degradation pathways of isovalerylspiramycin III (C) in the simulated gastric fluid.

Table 2
Summary of degradation products and rates of degradation of spiramycin derivatives in simulated gastric fluid



	k (h^{-1})	$t_{1/2}$ (h)	Products	m/z	Identification	R_1	R_2	R_3
A	0.74	0.94	A_1	699	Neospiramycin I	H	–	$C_8H_{16}NO$
			A_2	786	Leucomycin A1	H	$COCH_2CH(CH_3)_2$	H
			A_3	558	Forocidin I	H	–	$C_8H_{16}NO$
B	0.63	1.09	B_1	741	Neospiramycin II	$COCH_3$	–	$C_8H_{16}NO$
			B_2	828	Josamycin	$COCH_3$	$COCH_2CH(CH_3)_2$	H
			B_3	600	Forocidin II	$COCH_3$	–	$C_8H_{16}NO$
C	0.58	1.20	C_1	755	Neospiramycin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
			C_2	842	Platenomycin A1	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$	H
			C_3	614	Forocidin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
D	0.70	0.99	D_1	755	Neospiramycin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
			D_2	828	Deforosaminebutanoylspiramycin III	$COCH_2CH_3$	$COCH_2CH_2CH_3$	H
			D_3	614	Forocidin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
E	0.77	0.90	E_1	755	Neospiramycin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
			E_2	814	Midecamycin A1	$COCH_2CH_3$	$COCH_2CH_3$	H
			E_3	614	Forocidin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
F	0.78	0.89	F_1	755	Neospiramycin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
			F_2	800	Deforosamineacetylspiramycin III	$COCH_2CH_3$	$COCH_3$	H
			F_3	614	Forocidin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
G	2.24	0.31	G_1	755	Neospiramycin III	$COCH_2CH_3$	–	$C_8H_{16}NO$
			G_2	614	Forocidin III	$COCH_2CH_3$	–	H
H	2.67	0.26	H_1	741	Neospiramycin II	$COCH_3$	–	$C_8H_{16}NO$
			H_2	600	Forocidin II	$COCH_3$	–	H
I	3.47	0.20	I_1	699	Neospiramycin I	H	–	$C_8H_{16}NO$
			I_2	558	Forocidin I	H	–	H

$C_8H_{16}NO$ represents the forosamine group.

Thus, introduction of the isovaleryl group at position 4″ alters the degradation pathway. The degradation pathways of isovalerylspiramycin I and II were similar to those of isovalerylspiramycin III.

3.1.3. Degradation products of butanoylspiramycin III (D), propanoylspiramycin III (E) and acetylspiramycin III (F)

The degradation pathways of these compounds were similar to those of isovalerylspiramycin III. The results are given in Table 2. It was concluded that loss of forosamine followed by loss of mycarose is the predominant pathway of decomposition, based on the larger abundance of the deforosamine degradation product relative to the demycarose degradation product in the full scan mass spectra. The acyl group at position 3 was found to have little effect on the degradation pathway, whereas an acyl group at position 4″ can change the degradation pathway of spiramycin derivatives.

Most of the deforosamine degradation products arising from primary loss of forosamine from bitespiramycin are 16-member macrolide antibiotics such as platenomycin A1, josamycin and leucomycin A1 (Table 2), which have been used as therapeutic agents for decades. The antibiotic activity of products produced by loss of mycarose were reported to be lower than that of spiramycin [12]. Primary loss of forosamine is the predominant degradation pathway for 4″-acylated spiramycin derivatives and results in the superior clinical efficacy of bitespiramycin.

3.2. Kinetic study of spiramycin derivatives in simulated gastric fluid

As reported in our previous paper, spiramycins I–III (using mobile phase A) or isovalerylspiramycins I–III (using mobile phase B) were determined simultaneously within 6.5 min [15]. In the current study, the LC/MS^n method re-

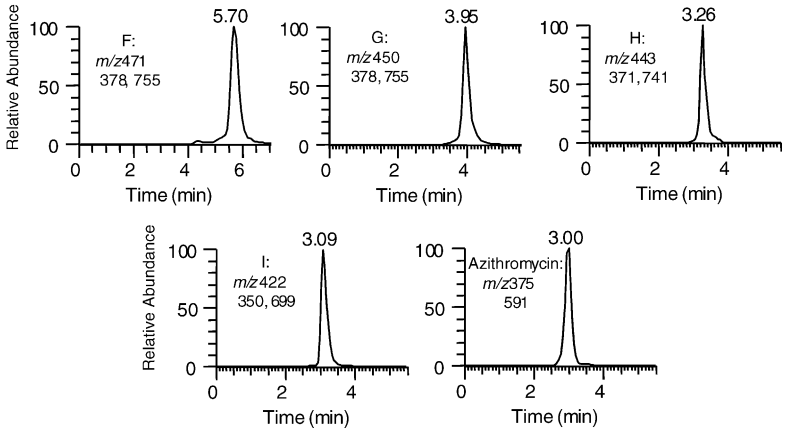


Fig. 5. SRM chromatograms of spiramycins I (I), II (H), III (G), acetylspiramycin III (F) and internal standard (azithromycin) using mobile phase A.

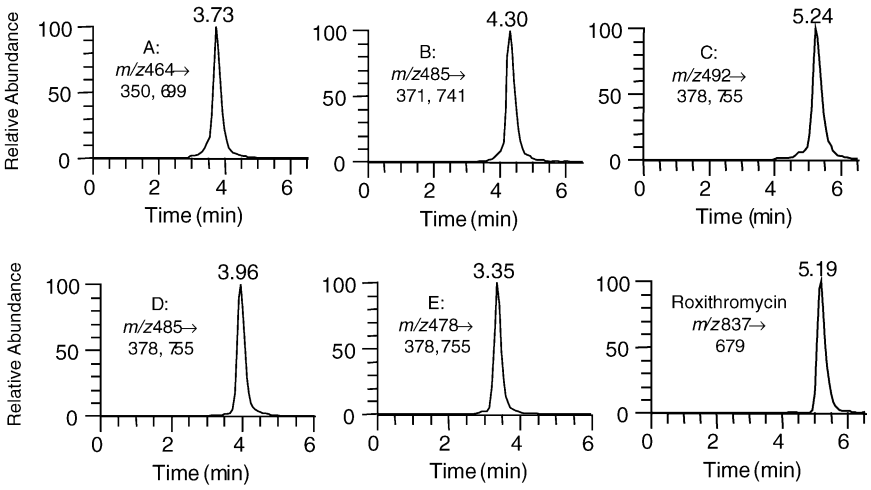


Fig. 6. SRM chromatograms of isovalerylspiramycins I (A), II (B), III (C), butanoylspiramycin III (D), propanoylspiramycin III (E), and internal standard (roxithromycin) using mobile phase B.

quired the determination of nine spiramycin derivatives and used a slightly optimized extraction procedure. Typical chromatograms for the spiramycin derivatives and internal standards using the two mobile phases are shown in Figs. 5 and 6.

The correlation coefficients (r) ranged from −0.991 to −0.997. The mean k and half-lives ($t_{1/2}$) of spiramycin derivatives are given in Table 2. The acyl group at position 3 has a minimal effect on the rate of decomposition of spiramycin derivatives in acid based on the similarity in rate constants for spiramycins I–III and isovalerylspiramycins I–III. The order of acid-stability for both sets of compounds is I < II < III.

As shown in Table 2, the degradation rates of 4″-acylated spiramycin derivatives (A–F) are about three- to four-fold less than those of spiramycins I–III (G–I). By comparing the k and $t_{1/2}$ values for C–G (where the groups at position 3 are identical), it is clear that the longer the side chain of the acyl group at position 4″, the more stable the spiramycin derivative in acid solution. Thus, the fact that 4″-acylated spiramycin derivatives are better absorbed from the gastrointestinal tract than spiramycin [7] may be partly due to their greater acid-stability.

4. Conclusions

The main degradation products and degradation rates of spiramycin derivatives in simulated gastric fluid have been characterised by LC/MS^n. An acyl group at position 3 has only a small effect on the acid-catalysed degradation of spiramycin derivatives whereas an acyl group at position 4″ enhances the acid-stability of spiramycin derivatives and alters their degradation pathway such than loss of forosamine rather than loss of mycarose becomes the major degradation pathway.

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References

[1] S. Kernbaum, J. Antimicrob. Chemother. 16 (1985) 199–200.
[2] E. Rubinstein, N. Keller, J. Antimicrob. Chemother. 42 (1998) 572–576.
[3] R.T. Rocha, C.E. Awad, A. Ali, R. Matyas, A.C. Vital, C.O. Silva, Int. J. Clin. Pract. 53 (1996) 433–436.
[4] P. Mourier, A. Brun, J. Chromatogr. B 704 (1997) 197–205.
[5] L. Liu, E. Roets, J. Hoogmartens, J. Chromatogr. A 764 (1997) 43–53.
[6] G.D. Shang, J.L. Dai, Y.G. Wang, J. Antibiot. (Tokyo) 54 (2001) 66–73.
[7] X.G. Shi, D.F. Zhong, Asian J. Drug Metab. Pharmacokinet. 3 (2003) 134–137.
[8] S. Nakazawa, M. Shimoyama, A. Maeda, M. Matsubayashi, J. Antibiot. (Tokyo) 20 (1967) 259–261.
[9] S. Omura, M. Katagira, I. Umezawa, K. Komiyama, T. Maekawa, K. Sekikawa, A. Matsumae, T. Hata, J. Antimicrob. 11 (1968) 532–538.
[10] S. Omura, H. Sano, T. Sunazuka, J. Antimicrob. Chemother. Antibiotics 16 (1985) 1–11.
[11] H. Sano, T. Sunazuka, H. Tanaka, K. Yamashita, R. Okachi, S. Omura, J. Antibiot. (Tokyo) 37 (1984) 760–772.
[12] W.Z. Feng, P.Y. Qi, T. Zhou, Y. Miao, X.B. Duan, Acta Pharm. Sin. 32 (1997) 934–937.
[13] K. Ramu, S. Shringarpure, J.S. Williamson, Pharm. Res. 12 (1995) 621–629.
[14] R.A. Rourick, K.J. Volk, S.E. Klohr, T. Spears, E.H. Kerns, M.S. Lee, J. Pharm. Biomed. Anal. 14 (1996) 1743–1752.
[15] D.F. Zhong, X.G. Shi, L. Sun, X.Y. Chen, J. Chromatogr. B 791 (2003) 45–53.