Study of Erythromycin A Decomposition Products in Aqueous Solution by Solid-phase Microextraction/Liquid Chromatography/Tandem Mass Spectrometry

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The decomposition of erythromycin A (EA) in aqueous solution was examined in the pH range 2–13 by means of combined solid-phase microextraction (SPME) and liquid chromatography/electrospray ionization tandem mass spectrometry. Degradation of EA, especially at lower pH values (pH $\leq$ 3), was very rapid and yielded a wide variety of decomposition products. Identification of these degradation products was achieved by means of tandem mass spectrometry in the product ion and precursor ion scan modes. Anhydroerythromycin A was shown to be the major reaction product in both acidic and basic solutions. Among the different SPME fibers investigated for extraction from aqueous solutions, polydimethylsiloxane/divinylbenzene exhibited the best performance for EA and its degradation products.
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Received 18 November 1997; Revised 25 November 1997; Accepted 26 November 1997
Rapid Commun. Mass Spectrom. **12**, 123–129 (1998)

Erythromycin A is a macrolide antibiotic produced by a strain of *Streptomyces erythreus*. Its structure (Fig. 1) consists of a 14-membered lactone ring (erythronolide A), a 9-oxo group and two attached sugar residues (desosamine and cladinose).[1] Erythromycin A is extensively used in the treatment of bacterial infections in both human and veterinary practice[2] as well as in other areas such as aquaculture.[3] The decomposition of erythromycin A has been shown to destroy the antibiotic activity.[4] For example, when administered orally, erythromycin A undergoes dehydration *in vivo* under acidic conditions, resulting in formation of inactive metabolites.[5]

The present study reports results of a degradation study of erythromycin A (EA) in aqueous solution at both acidic and basic pH. The analytical methodology utilized here was a combination of solid-phase microextraction (SPME), fast short-column liquid chromatography (LC) and electrospray ionization (ESI) tandem mass spectrometry (MS/MS). We recently reported the successful application of SPME/LC/MS/MS to quantification of corticosteroids and steroid conjugates in urine,[6] and to analysis of *N*-methylcarbamate pesticides in water.[7] In this work, SPME/LC/MS/MS was applied to the analytical determination of EA and its decomposition products and to the characterization of the decomposition products by tandem mass spectrometry.

EXPERIMENTAL

Chemicals

Erythromycin A (purity approx. 98%), sodium hydroxide, hydrochloric acid and formic acid were obtained from Sigma-Aldrich (Mississauga, ON, Canada). Anhydroerythromycin A (AEA) was kindly provided by Dr. Michael Quilliam (IMB/NRC, Halifax, Nova Scotia). Acetonitrile, methanol (Caledon, Georgetown, ON, Canada) and Milli-Q water (Millipore, Bedford, MA, USA) were used as solvents.

Degradation experiments

Aqueous test solutions of varying pH were prepared by adjusting the pH of deionized water with formic acid or 1.0 M sodium hydroxide. To investigate a possible influence of the pH modifier on the degradation reactions, additional experiments at pH 3.0 with hydrochloric acid were conducted and compared to the corresponding experiments with formic acid at pH 3.0. Degradation was carried out at room temperature (22 °C) in brown 4 mL glass vials. The solutions (3 mL) were continuously stirred during the incubation period with a magnetic micro-stirring bar. At predetermined times, 20 μL of the reaction mixtures were withdrawn (and immediately analyzed by LC/MS/MS); otherwise SPME/LC/MS/MS was directly performed in the reaction vials. Three replicate experimental series were conducted at each pH.

Solid-phase microextraction and liquid chromatography

Details of the SPME/LC parameters are described elsewhere.[6,7] Briefly, a SPME/LC interface from Supelco (Bellefonte, PA, USA) was used in this study. Three types of fibers were evaluated: 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB), 85 μm polyacrylate (PA) and 50 μm carbowax/templated resin (CW/TPR). For sample extraction, the entire fiber was immersed in the reaction mixture for 15 minutes. Subsequently, the fiber was inserted into the desorption chamber of the SPME/LC interface, which was previously filled with a solution of methanol/water = 50:50 (v/v). Analyte desorption was achieved in the static mode over a period of 5 min. After desorption, the entire contents of the desorption chamber were flushed directly onto the HPLC column by means of

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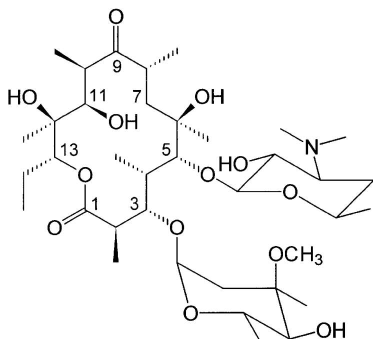

Figure 1. Chemical structure of erythromycin A.

the mobile phase gradient flow. Separations were achieved using a Hewlett-Packard (Palo Alto, CA, USA) model 1090 HPLC system with short columns (50 × 4.0mm) packed with 3 μm C-8 particles (YMC, Wilmington, NC, USA). Gradient elution was applied using acetonitrile/water (5:95 [v/v] + 0.1% formic acid to 85:15 within 15 min) at a flow-rate of 1.0 mL/min. Aliquots of 10 μL of reaction mixtures and standard solutions were injected by a HP 1090 autosampler.

Electrospray ionization tandem mass spectrometry

Electrospray data were acquired using an API 300 (MDS-Sciex, Concord, ON, Canada) triple-quadrupole mass spectrometer ($Q_1q_2Q_3$). A spray voltage of 5 kV, a ring-electrode voltage of 320 V and an orifice-skimmer potential difference of 40 V were used. The mobile-phase flow was split, delivering ~60 μL/min to the mass spectrometer. Q_1 was scanned at a rate of 0.5 s/scan over the range *m/z* 500–800 for full-scan or precursor ion scan experiments. MS/MS in the product and precursor ion modes was performed in q_2 using nitrogen as collision gas at a pressure of 2.5 mTorr (collision gas thickness [CGT], 1.65×10^{15} atoms $\times$ cm^{-2}). The collision-offset voltage, ΔV_c, which determines the laboratory frame collision energy, was set to 25 V. (ΔV_c refers to the potential difference between the high pressure entrance quadrupole lens q_0 and the collision cell quadrupole q_2).

RESULTS AND DISCUSSION

LC separation at low pH

One of the objectives of this investigation was to develop a fast LC/MS method for the analytical determination of erythromycin A and its decomposition products. Separations were conducted on short C-8 reversed-phase columns, similar to those recently used for the separation of antibiotics and pesticides.[6–8] Out of concern for possible on-column degradation of EA, most literature LC methods for EA used only slightly acidic, neutral or even mildly basic mobile phase conditions.[9,10] The reversed-phase LC columns employed nowadays for efficient separations of pharmaceutical drugs, however, often require acidic mobile phases for optimum performance. Since EA decomposition products were the target group in this study, the problem of on-column degradation at low pH had to be addressed prior to investigation because on-column degradation products formed during the analytical separation could falsely be assigned as products of the actual decomposition study.

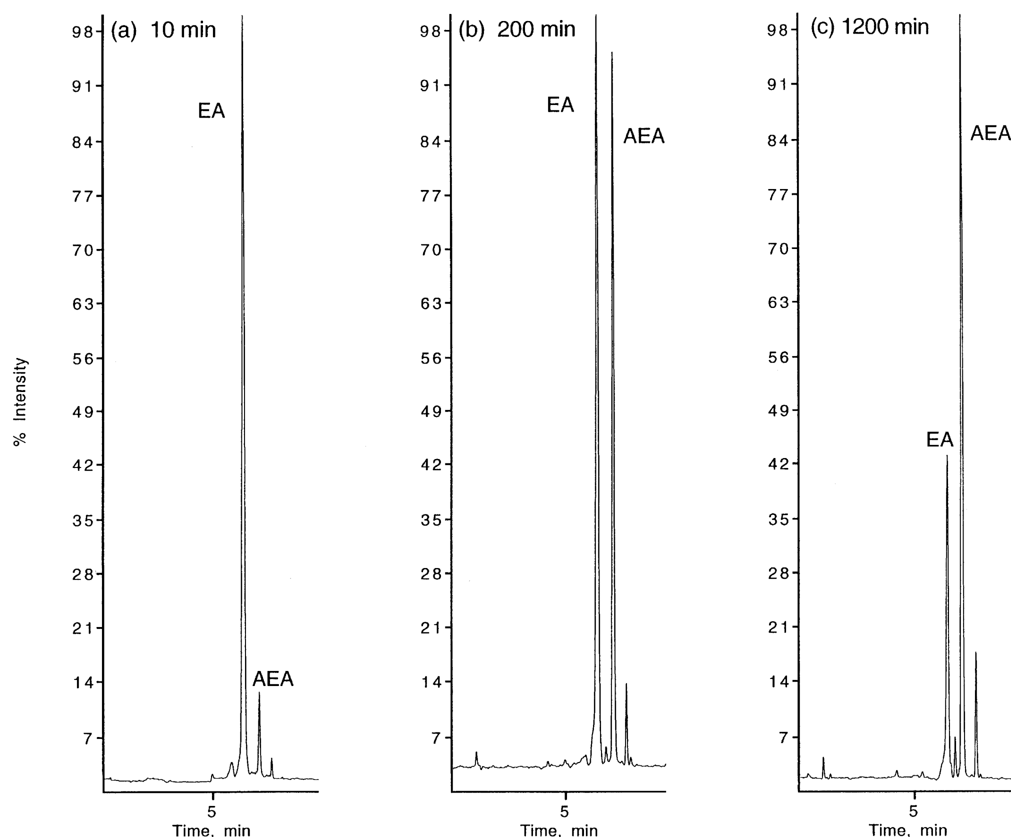

Figure 2. Electrospray full-scan chromatograms (*m/z* 500–800) of EA and its decomposition products after various incubation periods at pH 3.5.

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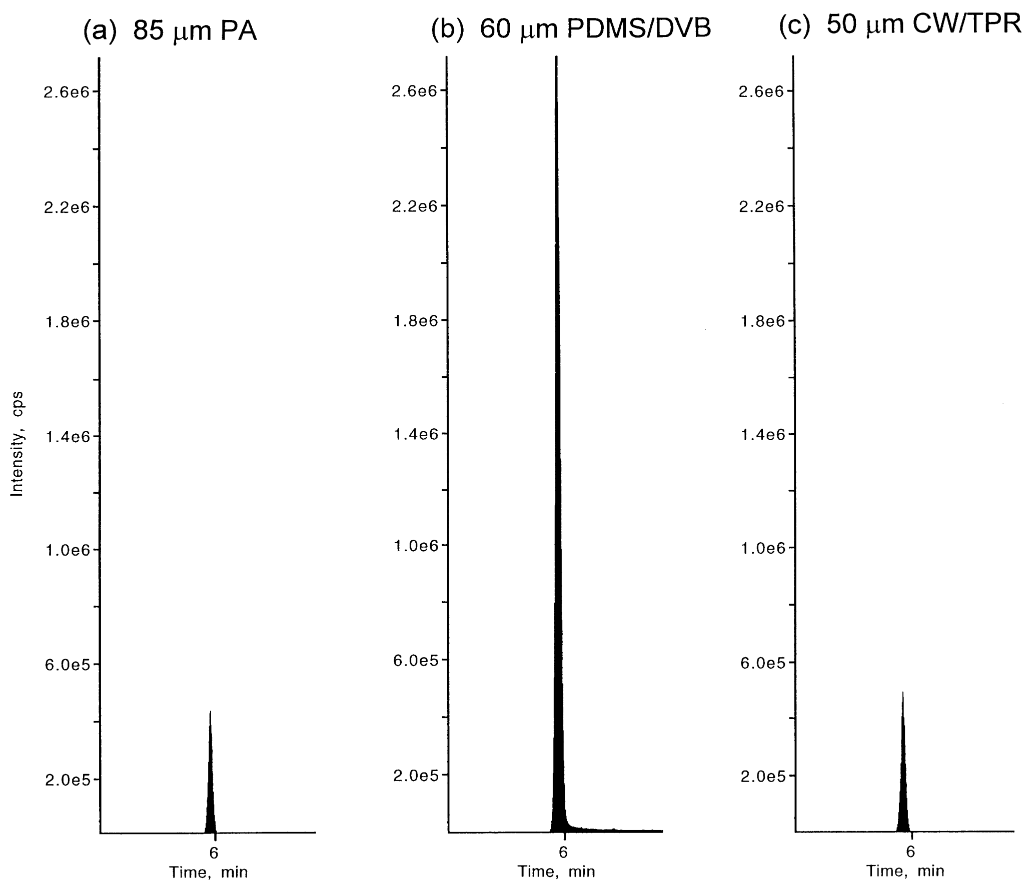

Figure 3. Evaluation of three extraction fibers for the SPME/LC/MS analysis of EA in water ($c_o = 2$ μg/mL): (a) 85 μm PA, (b) 60 μm PDMS/DVB, (c) 50 μm CW/TPR. Partial SIM chromatograms of the MH^+ ion (*m/z* 734).

In acidic medium, anhydroerythromycin A (AEA) is the major degradation product of EA (see below). Pleasance *et al.*[11] did not observe any evidence for formation of AEA, nor of other degradation products, at low pH on the time-scale of their chromatography in the LC/MS analysis of EA with an acetonitrile/water mobile phase plus 0.2% formic acid. Under the chromatographic conditions used in this study (acetonitrile/water + 0.1% formic acid, see experimental section), again virtually no degradation of pure EA standard was observed during chromatography (<1%). The small extra peaks detected in the chromatogram (e.g. erythromycin B, C and D [EB,EC,ED]) were shown to be present in the original EA standard by comparison with an LC separation at pH 7.0. Interestingly, however, when exposing EA to an acetonitrile/water (50/50, v/v) + 0.1% formic acid mixture for *ca.* 6 min (approx. retention time of EA on the column used) prior to injection onto the LC column, significant formation of AEA was observed in the subsequent LC/MS analysis. A possible explanation for this discrepancy can be given based on the assumption that degradation takes place only when EA is in the free solution of the mobile phase, and not when it is bound to the stationary phase. That is, the effective degradation time then corresponded to the dead time (void volume) of the separation column, which was less than 30 seconds under the chromatographic conditions applied here. Only minor degradation could be expected at such short degradation times at the pH of the mobile phase (pH 2.7).

Further experiments are necessary to confirm this hypothesis. Figure 2 shows typical chromatograms of a

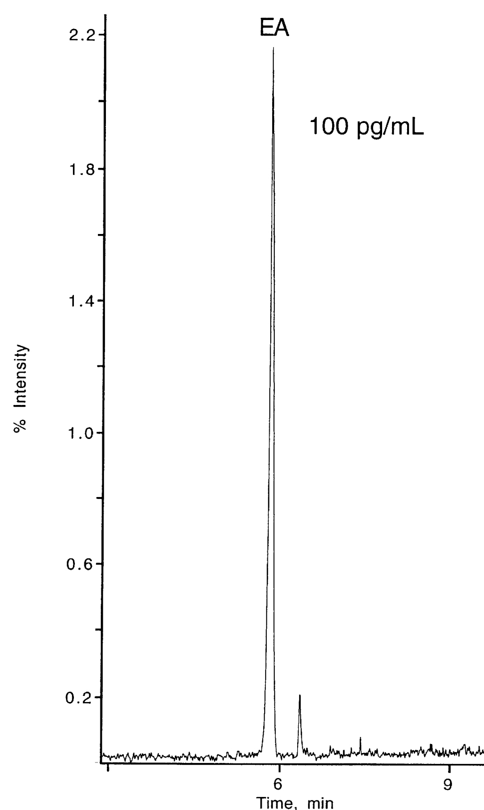

Figure 4. SPME/LC/MS analysis of a water sample spiked with EA at the 100 pg/mL level at pH 7.0 after an incubation period of 90 min. SIM chromatogram of the MH^+ ion (*m/z* 734).

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Table 1. Observed pseudo-first order rate constants k (min^{-1}) for the decomposition of EA in water as a function of pH at 22 °C[a]

pH	k (min^{-1})
2.0	2.36×10^{-1}
3.0	1.30×10^{-2} (1.02×10^{-2})[b]
4.0	1.80×10^{-3}
10.0	5.67×10^{-5}
12.0	1.83×10^{-3}
13.0	1.56×10^{-2}

[a] $c_o = 2$ μg/mL. pH adjustment with formic acid ($n = 3$).
[b] The rate constant in parentheses indicates the pseudo-first order rate constant observed by adjusting to pH = 3.0 with hydrochloric acid instead of formic acid.

reaction mixture at pH 3.5, for various incubation times but otherwise identical conditions

SPME/LC method optimization

A recent paper by Coleman[12] describes a very detailed comparison study of the extraction behavior of two different fibers for SPME/GC analysis of Maillard reaction products. In the present study, three different fibers were evaluated for EA extraction from aqueous matrices: 85 μm PA, 60 μm PDMS/DVB, and 50 μm CW/TPR (see experimental section).

Figure 3 shows a performance comparison of these fibers for EA in water under identical experimental conditions. The PDMS/DVB fiber exhibited superior extraction efficiency as compared to the PA and CW/TPR fibers. This result was somewhat unexpected, since in recent experimental investigations of steroids and carbamates,[6,7] the CW/TPR fibers had shown far better performance for those analytes than the PA and PDMS/DVB fibers. Electrolytes are routinely added to the samples in SPME experiments to enhance extraction efficiencies.[6,7] In the experiments described in this study, however, no salt (NaCl) was added out of concern for a possible influence on the degradation reactions.

A preliminary validation of the SPME/LC/MS method for EA using the PDMS/DVB fiber was conducted. The method

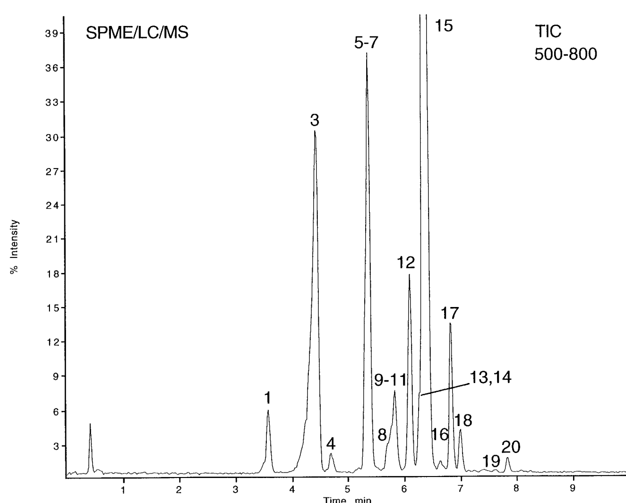

Figure 5. SPME/LC/MS analysis of EA decomposition products after a 24 h incubation period at pH 3.0 (full-scan chromatogram, *m/z* 500–800).

was shown to be linear over at least 3 orders of magnitude in the concentration range 1 ng/mL to 2 μg/mL, with precision values <10% RSD. The detection limit for EA, obtained using the selected ion monitoring (SIM) mode of the mass spectrometer, was very low, in the lower pg/mL range in water (Fig. 4). Interestingly, a comparison of the absolute EA amounts analyzed by both direct injection of aqueous EA standard solution (extrapolated to 200 μL injection volume which corresponds exactly to the volume of the SPME/LC desorption chamber), and the amount extracted and transferred by SPME from an identical EA standard solution, revealed an approximately five times larger amount for the SPME extraction. That is, the enrichment factor of SPME for EA corresponded to about five.

A more detailed quantitative study of SPME extraction parameters for decomposition products of EA was not undertaken in this preliminary work due to the lack of appropriate analytical standards at the time of investigation. A complete evaluation of those extraction parameters will be reported separately as part of the ongoing study.

Influence of pH on degradation rates

The acid degradation of erythromycin A in aqueous solution is well characterized, and degradation mechanisms have been described in the literature.[1,2,13,14] It has been demonstrated that the major reaction product, anhydroerythromycin A, is formed via an equilibrium of EA and EA enol ether (EAEN) coupled to a direct conversion from EA to AEA.[1,13] In this study, the degradation at both acidic and basic pH has been investigated in the pH range 2–13 over an incubation period of up to 48 hours.

Pseudo-first order rate constants k (min^{-1}) were calculated from the percent degradation versus incubation time curves for pH = 2, 3, 4, 10, 12 and 13 (see Table 1) at EA concentrations of $c_o = 2$ μg/mL. The quantification of the remaining EA in solution was based on peak area measurements in the *m/z* 734 ion chromatograms. Investigation of pH values >4 and <10 resulted in degradation yields of less than 5% within the investigated incubation period range (48 hours). Therefore, rate constants were not determined in this pH range. Also, the pH ranges <4 and >12 were of particular interest here, because identification of decomposition products was the primary goal of this study. Moreover, as will be shown in the next section, at low pH (pH < 3) a larger number of different decomposition products was formed as compared to experiments at high pH.

Identification of decomposition products

In the following discussion, emphasis was laid on identification of decomposition products formed at low pH. Differences from degradation in basic media are described at the end of this section. The tentative identification of MH^+ ions of decomposition products was initially achieved by means of full-scan analyses of the reaction mixtures. One such SPME/LC/MS analysis is shown in Fig. 5. Table 2 lists the peak numbers together with retention times, as well as the *m/z* values of the identified MH^+ ions.

Characterization of the tentatively identified decomposition products was accomplished by collision-induced dissociation (CID) of the MH^+ ions in the collision cell of a triple-quadrupole mass spectrometer. For example, the

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Table 2. Summary of SPME/LC/MS/MS data for degradation of erythromycin A in acidic medium (incubation period, 0–24hr)

Peak no.	t_r (min)	MH^+ (*m/z*)	PIC[a] (158^+)	Major product ions (*m/z*)	Tentative identification
1	3.60	592	*	574, 556, 538, 158, 116	EANO–cladinose
2	4.35	750		-[b]	?
3	4.45	576	*	558, 540, 522, 158, 116	EA–cladinose
4	4.71	766	*	-[b]	?
5	5.15	720	*	576, 558, 158	EC
6	5.30	574	*	560, 158	?
7	5.38	558	*	540, 522, 408, 233, 158, 116	EA-cladinose-H_2O
8	5.70	556	*	-[b]	?
9	5.78	734	*	716, 698, 576, 558, 540, 522, 500, 316, 158, 116	EA
10	5.85	748	*	558, 158	?
11	5.90	704		686, 576, 560, 542, 524	ED
12	6.06	702	*	558, 540, 522, 158	AEC
13	6.25	702	*	558, 540, 522, 158	?
14	6.27	702		544, 526, 144	AdMeEA, dMeEAEN
15	6.35	716	*	698, 558, 540, 522, 500, 482, 464, 158	AEA
16	6.61	718	*	560, 542, 158, 116	EB
17	6.82	716	*	558, 540, 522, 500, 482, 158	EAEN
18	7.00	716	*	558, 158	EA-H_2O
19	7.55	700		542, 524	AEB
20	7.82	698	*	540, 522, 158	AEA-H_2O?

[a] Confirmation of MH^+ ions of decomposition products containing the desosamine residue by precursor ion scanning (*m/z* 158).
[b] The concentration of these decomposition products in the mixtures was too low to obtain an interpretable CID spectrum.

CID spectrum of EA (MH^+ at *m/z* 734; see Table 2) exhibited ions corresponding to neutral losses of water (*m/z* 716, 698) and neutral loss of cladinose with simultaneous hydrogen transfer[11] (*m/z* 576) and subsequent water losses (*m/z* 558, 540, 522). In addition, an intense ion at *m/z* 158 was observed under CID which was due to the dehydroform of the protonated desosamine. This general fragmentation scheme was observed with almost all EA decomposition products. Interestingly, compounds related to EA containing the intact desosamine residue could therefore readily be detected in the precursor ion scan mode of the tandem mass spectrometer, by monitoring *m/z* 158 in Q_3 while scanning the Q_1 unit. Such precursor ion scans suggested the presence of at least 15 components which gave rise to the product ion at *m/z* 158 (Fig. 6). The MH^+ ions identified in this manner confirmed most of the tentatively assigned protonated molecule ions from the full-scan analyses (Table 2).

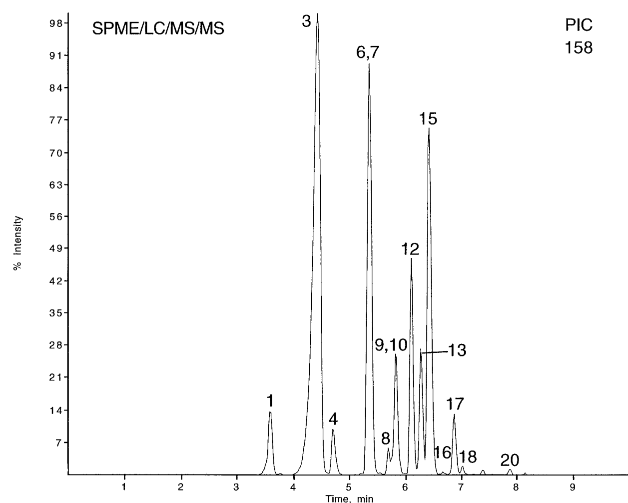

Figure 6. SPME/LC/MS/MS analysis of EA decomposition products after a 24 hr incubation period at pH 2.2 (precursor ion chromatogram [PIC] of *m/z* 158; $c_o = 2$ µg/mL).

Commercial standards of erythromycin A (peak **9**, Table 2) usually contain traces of EB, EC and ED. Consequently, EB, EC and ED (peaks **16, 5** and **11,** respectively) and their

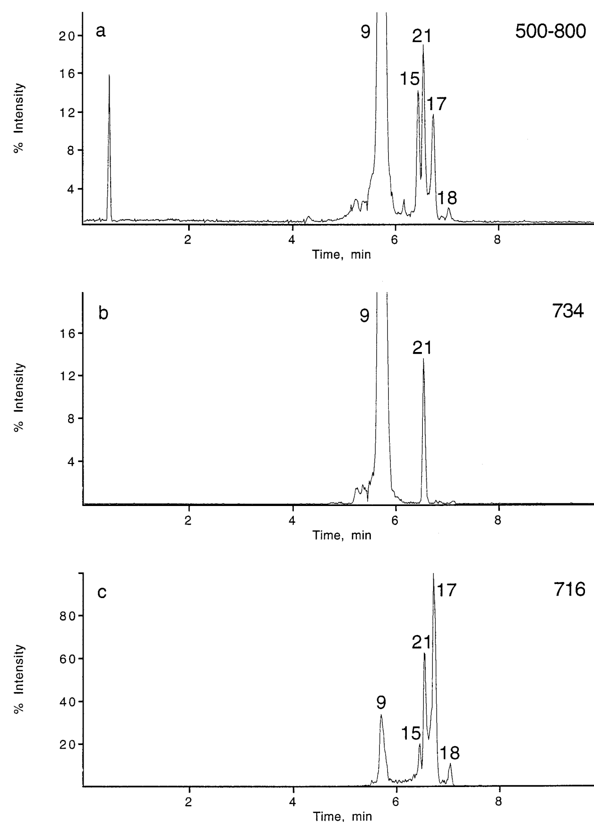

Figure 7. LC/MS separation of EA decomposition products after a 30 min incubation period at pH 12.0 ($c_o = 2$ µg/mL). (a) Full-scan trace (*m/z* 500–800), (b) ion trace of *m/z* 734, (c) ion trace of *m/z* 716.

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subsequent degradation products (peaks **12** and **19**) were also observed as minor peaks in our analyses.

Major decomposition products at low pH were detected as protonated molecules at *m/z* 716, 702, 592, 576 and 558 in the full-scan analyses as well as in the precursor ion chromatograms. Three peaks were observed in the *m/z* 716 chromatograms. The major *m/z* 716 peak (**15**) was shown to be AEA by comparison of retention time and CID spectrum with that of a pure standard. The second peak in the *m/z* 716 trace (**17**), eluting after AEA, was assigned to EA enol ether based on the degradation pathway described in the previous section and similar results reported by Pleasance and co-workers.[11] No standard, however, was available to confirm this tentative assignment. The exact structure of the last *m/z* 716 peak (**18**) is presently unknown. The product ion spectrum (Table 2) of this minor peak, however, suggests an isomer of AEA.

At least three peaks (**12–14**) were observed in the *m/z* 702 chromatograms. Possible compound assignments for these peaks include anhydroerythromycin C (AEC), anhydro-*N*-demethylerythromycin A (AdMeEA) and *N*-demethylerythromycin A enol ether (dMeEAEN).[11] AEC (peak **12**) was likely to have been formed by degradation of the EC impurity present in the standard (see above). Its tentative identification was supported by a neutral loss of 144 Da under CID, confirming that the neutral sugar was demethylcladinose rather than cladinose (158 Da), characteristic for EC (peak **5**) or EC decomposition products. Peak **13** most likely corresponds to an isomer of AEC, because of its product ion spectrum which is identical to that of **12**. Peak **14** probably is an *N*-demethyl compound such as AdMeEA or dMeEAEN because its product ion spectrum did not exhibit a fragment ion at *m/z* 158 (desosamine), but rather an intense ion at *m/z* 144 (demethyl-desosamine) and a neutral loss of 158 Da (cladinose).

Peaks **3** and **7** were assigned to degradation products resulting from loss of cladinose (peak **3**) and cladinose plus water from EA (peak **7**). In the CID spectra of both, the signals at *m/z* 158 indicate intact desosamine residues bound to the macrolide ring. Similarly, **4, 6, 8** and **10** exhibited *m/z* 158 in their spectra upon CID of the respective MH^+ ions. The identity of these products in the reaction mixtures, however, remains unclear.

At short incubation times (<30 min) a very small peak at *m/z* 750 (peak **2**) was observed in the chromatograms at pH < 4. Unfortunately, the concentration of this compound was too low to obtain an interpretable CID spectrum. This *m/z* 750 peak was not observed as an impurity in the EA standard chromatograms and therefore must have been formed via degradation of EA. Known isomers related to EA with a nominal mass of 749 are erythromycin F (EF) and erythromycin A *N*-oxide (EANO). Both these possibilities were ruled out, however, because EF is not likely to be formed as a degradation product of EA, and EANO was shown to elute after EA under very similar chromatographic conditions.[11] Interestingly, however, peak **1** (MH^+ at *m/z* 592) could possibly indicate the (transient) presence of EANO in the degradation mixtures. That is, product **1** may be produced by initial formation of EANO and subsequent degradation to **1** via loss of cladinose. The CID spectrum of peak **1** (*m/z* 592) is consistent with such an assignment.

The last eluting peak in the chromatograms (MH^+ at *m/z* 698, peak **20** was assigned as a product resulting from loss of water from AEA. The product ion spectrum of *m/z* 698 suggests intact desosamine (*m/z* 158) and cladinose (158 Da neutral loss) residues at the macrolide ring. No conclusive information about the structure of the macrolide moiety, however, could be obtained from the CID analysis. In general, as was also observed by Pleasance and co-workers,[11] the product ion spectra of all decomposition products of EA observed in this study were consistent with the assignments in Table 2, but did not provide unequivocal proof of identity under low energy CID conditions.

As described in the previous section, degradation at high pH was slower than at low pH. In general, however, quite similar products were formed upon degradation. The major base degradation product was AEA, as was observed in acidic media. Other similarities include the formation of decomposition products corresponding to peaks **1, 3, 4, 6, 7, 13, 17, 18** and **20** (Table 2) plus the peaks corresponding to impurities present in the original EA standard. Two interesting differences in degradation behavior, however, should be pointed out here. First, a new compound with an MH^+ ion at *m/z* 752 was observed, eluting at approximately 5.4 min. This quite intense peak was not observed in the chromatograms of EA reaction mixtures with pH values <10.

Second, an additional peak at *m/z* 734 was observed in the chromatograms (peak **21**, Fig. 7), eluting *ca.* 1 min after EA. This peak was not obtained at low pH, but was also formed at neutral pH (see, for example, Fig. 4). This compound could possibly have resulted from ring contraction of EA and translactonization to the pseudo-erythromycin A series, as shown by Cachet *et al.*[15] and Kibwage and co-workers.[16] Peak **21** was detected only at short incubation periods. It disappeared almost completely from the mixtures at degradation times >60 min. Such an isomerization could also explain a phenomenon that occurred when analyzing reaction mixtures of EA at high pH values (pH > 12) after long incubation periods (>20 hr). In those chromatographic runs, substantial peak broadening for the EA peak was obtained, which could indicate on-column re-isomerization of the pseudo-erythromycin series back to the regular erythromycin series when exposed to the acidic conditions of the mobile phase.[16] Further studies are necessary to confirm these findings.

CONCLUSIONS

This study clearly illustrates the suitability of SPME/LC/MS for the analysis of erythromycin A and its decomposition products in aqueous solution. The instrumental sensitivity of this technique was shown to be in the lower picogram range for erythromycin A. The sensitivity could be even further improved by enhancing the effectiveness of the SPME extraction step via addition of salt (NaCl) to the sample solutions (increases in SPME extraction efficiencies of up to 23 times those without salt were recently reported for steroids[6] and pesticides[7]).

The rate of decomposition of erythromycin A was shown to be strongly dependent on pH. Erythromycin A was relatively stable at pH values >4 and <10, but became increasingly labile at both low and very high pH values, especially at pH $\leq$ 3. In both acidic and basic media, anhydroerythromycin A was observed as the major degradation product.

The high selectivity of the present method was exhibited by results obtained by the combined application of precursor ion and product ion scans for partial structural characterization and identification of decomposition products. Unequi-

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vocal proof of identities, however, was often not possible with the triple-quadrupole LC/MS/MS instrument used in this study because of the relatively simple low energy CID spectra obtained for EA derivatives. MS^n experiments, e.g. with an ion-trap mass spectrometer and suitable analytical standards for decomposition products, would greatly support the identification procedure.

Acknowledgements

The authors wish to thank Dr. Robert K. Boyd and Dr. Archibald W. McCulloch (Institute for Marine Biosciences, Halifax, Nova Scotia) for their valuable discussions and suggestions in the preparation of this manuscript.

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