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ORIGINAL PAPER

Combined use of liquid chromatography with mass spectrometry and nuclear magnetic resonance for the identification of degradation compounds in an erythromycin formulation

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Abstract A commercial erythromycin formulation containing erythromycin A (EA) as the major compound showed the presence of an unknown degradation compound that was co-eluted with erythromycin E (EE) in the European Pharmacopoeia (Ph. Eur.) liquid chromatographic (LC) method. The amount of the degradation compound increased with respect to time. To separate this unknown (UNK1), investigation was performed with different LC methods coupled to ultraviolet detection (LC-UV). With the present Ph. Eur. method, the degradation compound could not be well separated. However, with the most selective LC-UV method (XTerra method), two more degradation products (UNK2 and UNK3) were found in the formulation which could not be observed using other methods because of their poor separation. By combining the results obtained with LC-UV, LC/MS and LC/NMR, the degradation products were identified as pseudoerythromycin A hemiketal (PsEAHK), erythromycin A enol ether carboxylic acid and erythromycin C enol ether carboxylic acid. PsEAHK is known to be a base-catalysed degradation product of EA, whereas the other two degradation products were newly identified.

Keywords Erythromycin · Degradation · LC-UV · LC/MS · LC/NMR

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Introduction

Erythromycin is a complex macrolide antibiotic mainly composed of erythromycin A (EA) and also of erythromycins B (EB), C (EC), D (ED), E (EE) and F (EF) [1–5]. Erythromycin has a bacteriostatic activity [6]. Chemically, it is a 14-membered macrolide with a 9-keto group, a neutral sugar and an amino sugar [7] (Fig. 1). It is produced by fermentation of a strain of *Streptomyces erythreus*. ED is the precursor to EB or EC, from which EA is formed, which in its turn is transformed into EF and EE. The contents of EB and EC are each limited to 5.0% in the European Pharmacopoeia (Ph. Eur.). Along with the aforementioned compounds, several other related substances may be formed by fermentation, e.g. EA N-oxide (EANO) [8], N-demethyl EA (N-deMeEA) [9], or by decomposition, e.g. anhydro EA (AEA), EA enol ether (EAEN) [10], pseudo EA enol ether (PsEAEN) and pseudo EA hemiketal (PsEAHK) [11, 12] (Fig. 2). EA degrades in both acidic and basic aqueous solutions. In acid-catalysed degradation, EA is in equilibrium with EAEN and is converted to AEA [13, 14]. Paesen et al. described the degradation of EA in neutral and alkaline solutions [15]. PsEAHK was found to be the main degradation product as the pH of the aqueous solution increased. PsEAEN was not detected in solutions above pH 9.0. On the other hand, Kim et

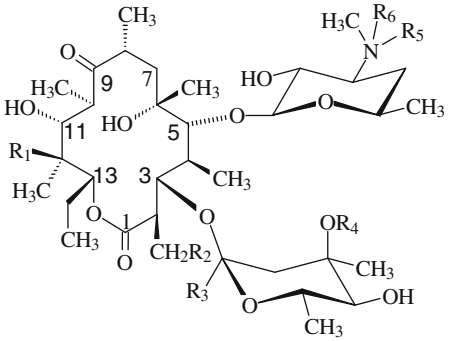


Fig. 1 Chemical structure of erythromycin A and related substances

	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A (EA)	OH	H	H	CH_3	CH_3	-
Erythromycin B (EB)	H	H	H	CH_3	CH_3	-
Erythromycin C (EC)	OH	H	H	H	CH_3	-
Erythromycin D (ED)	H	H	H	H	CH_3	-
Erythromycin E (EE)	OH	-O-		CH_3	CH_3	-
Erythromycin F (EF)	OH	OH	H	CH_3	CH_3	-
N-demethylerythromycin A (NdMeEA)	OH	H	H	CH_3	H	-
Erythromycin A N-oxide (EANO)	OH	H	H	CH_3	CH_3	O

al. described that the decomposition of EA to PsEAEN gradually increased along with the increment of pH from 7.0 to 9.0 [16]. They did not observe formation of PsEAHK; however, they did not show the capability of the chromatographic system used to separate PsEAHK.

As a result of the complex nature of erythromycin, a selective and sensitive method is necessary for quality control. An LC-UV method using a polymer column developed by Paesen et al. [17] is described in the Ph. Eur. [18]. In the Ph. Eur. monograph, all the aforementioned related substances except PsEAHK are included and identification can be done on the basis of the relative retention time (RRT). Several other LC-UV methods were described for the determination of erythromycin and its related substances. Only the most relevant ones of the last 10 years are mentioned here [19–23].

Amongst these, two LC methods were described using Astec (Astec method) [21] and XTerra columns (XTerra method) [22]. The XTerra method with a mobile phase consisting of acetonitrile, 0.2 M dipotassium hydrogen phosphate pH 7.0 and water was proved to be more suitable, because it is more selective towards the polar and small impurities [24]. The specified impurities of the Ph. Eur. and PsEAHK were located in the chromatogram. Using the structural elucidation power of LC/MS, we and others have identified several unknown impurities [25–28]. Pendela et al. coupled the XTerra method [22] to MS with a peak trapping technique. This technique facilitated the tracing of 25 impurities which were previously mentioned in the literature and also the identification of 13 new impurities [29]. Recently, the XTerra method was further improved by using a smaller particle size of the stationary phase and gradient elution [30].

A liquid erythromycin formulation (code name "sample ES") containing 4% of erythromycin and excipients was analysed using the Ph. Eur. method. An unknown compound (UNK1) was eluted before EA, but it was not well separated from EA and its corresponding peak overlapped with that of EE. However, UNK1 did not correspond to EE as (1) its retention time was slightly different from that of EE and (2) it was observed that the peak of UNK1 increased on standing, whereas EE is not formed by degradation. The RRT did not correspond to any other impurity mentioned in the monograph of the Ph. Eur. By increasing the concentration of *tert*-butanol in the mobile phase, UNK1 could be somewhat better separated from EA, but the separation from EE became worse. In order to be able to guarantee the quality of the product in terms of safety and efficacy, knowledge of the identity of degradation impurities is of the utmost importance. So, an investigation was carried out to better separate UNK1 by using three different LC-UV methods and to reveal its identity. In total three degradation products were found. Two of them are highly polar in nature and were not detected with the other methods because of poor separation. For further identification, LC was first hyphenated with MS. As this combination was not able to fully reveal the identity of the unknowns, NMR was also used.

Experimental

Instrumentation and chromatographic LC-UV conditions

Equipment 1 was used for the analyses carried out with the Ph. Eur. method and the Astec method. It consisted of an L-

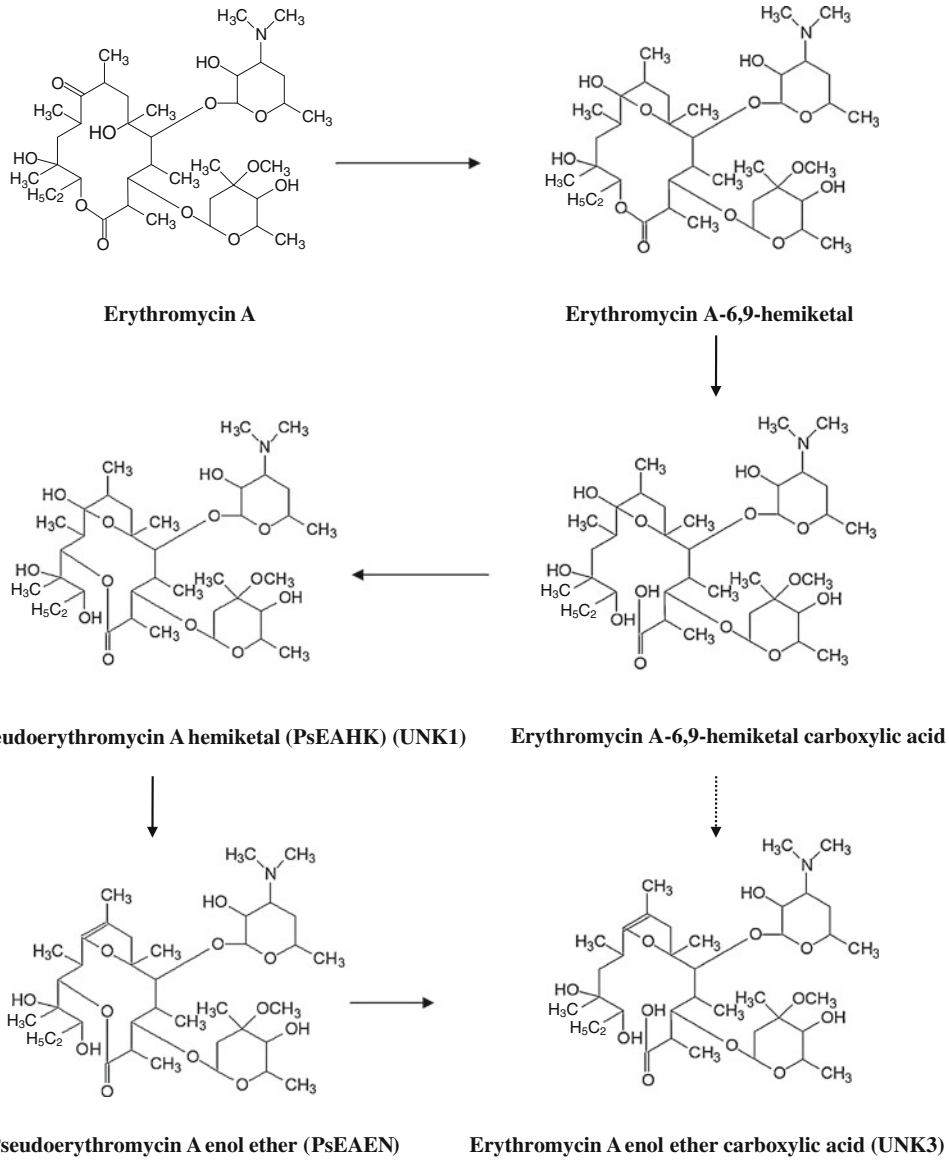


Fig. 2 Degradation pathway of erythromycin A

6200 Intelligent Pump, an autosampler Elite LaChrom Hitachi L-2200 and a UV detector Elite LaChrom L-2400 set at 215 nm (Merck-Hitachi, Darmstadt, Germany). As columns, a PLRP–S 8 μm, 1,000 Å, 250 mm×4.6 mm I.D. (Polymer Laboratories, Shropshire, UK) and an Astec C18 Polymeric 5 μm, 1,000 Å, 250 mm×4.6 mm I.D. (Alltech, Whippany, NJ, USA) were used for the Ph. Eur. and Astec method, respectively.

Equipment 2 was used for the analyses carried out with the XTerra method. It was composed of a Spectra System P1000 XR pump, Spectra Series AS 100 autosampler, a linear UV-VIS 200 detector (Thermo Separation Products, San Jose, CA, USA) set at 215 nm and an XTerra RP C18, 3.5 μm, 250 mm×4.6 mm I.D. column (Waters, Milford, Massachusetts, USA). The temperature of the columns was maintained using a water bath with a heating circulator (Julabo EM, Seelbach, Germany) and Chromeleon software 6.60 (Dionex, Germering, Germany) was used for data acquisition.

In the Ph. Eur. method, the concentration of *tert*-butanol was increased from 16.5 to 20% to better separate UNK1 from EA. However, UNK1 was still only partially separated from EA and co-eluted with EE. For consistency of the methods, injection volume and detection wavelength were kept constant. The final chromatographic conditions of the three methods are summarized in Table 1.

LC instrumentation and chromatographic conditions for LC/MS

The LC apparatus consisted of a Spectra System1000XR quaternary pump, a Spectra Series AS 100 autosampler equipped with a 20-μL loop, a variable wavelength Spectra 100 UV-VIS detector set at 215 nm and ChromPerfect

Table 1 LC-UV conditions

	Ph. Eur. method	Astec method	XTerra method
Stationary phase	PLRP–S 8 μm, 1,000Å, 250×4.6 mm I.D.	Astec C18 Polymeric 5 μm, 1,000Å, 250×4.6 mm I.D.	XTerra RP C18, 3.5 μm, 250×4.6 mm I.D.
Mobile phase	Acetonitrile (3%, v/v), 0.2 M phosphate buffer pH 9.0 (5%, v/v), *tert*-butanol (20%, v/v), water (72%, v/v)	Acetonitrile (40%, v/v), 0.2 M phosphate buffer pH 9.0 (6%, v/v), water (54%, v/v)	Acetonitrile, 0.2 M K_2HPO_4, pH 7.0, water; mobile phase A (35:5:60, v/v/v); mobile phase B (50:5:45, v/v/v)
Elution	Isocratic	Isocratic	Gradient 0 till *x*: 100% A; *x* till (*x*+2): 100–0% A; (*x*+2) till (*x*+9): 0% A (*x*+9) till (*x*+10): 0–100% A; (*x*+10) till (*x*+20): 100% A; where *x* is the retention time (min) of EB
Injection volume (μL)	20	20	20
Column temperature (°C)	70	50	65
Flow rate (mL/min)	1.5	1.0	1.0
UV detection (nm)	215	215	215

4.4.23 software (Justice Laboratory Software, Fife, UK) for data acquisition. The LCQ (Thermo Finnigan, San Jose, CA, USA) ion trap mass spectrometer was equipped with an electrospray ionisation (ESI) source operated in the positive ion mode.

A volatile mobile phase containing acetonitrile/0.2 M ammonium acetate pH 7.0/water (270:100:630, v/v/v) was used at a flow rate of 1.0 mL/min. The XTerra RP C18 column (250×4.6 mm I.D.), 5 μm (Waters Corporation, Massachusetts, USA) was maintained at room temperature. Before entering into the MS, the mobile phase was split using a T-piece and 20% of the solution was sent into the MS.

LC instrumentation and chromatographic conditions for LC/NMR

The LC/NMR system consisted of a Varian ProStar 220 solvent delivery module (Varian Inc. CA, USA) equipped with a ProStar 430 AutoSampler (Varian Inc. CA, USA) and a ProStar 335 Photodiode Array Detector (Varian Inc. Australia). Off-line NMR and LC/NMR experiments were carried out on a 600 MHz Varian VNMRS spectrometer (Palo Alto, CA, USA) equipped with a dual 5-mm inverse-detection gradient (IDPFG) probehead for off-line, and a 60-μL active volume H{C,N} inverse-detection gradient flow cell (IFC) for LC/NMR experiments respectively. Standard pulse sequences for ^{1}H, ^{13}C, COSY, TOCSY, NOESY, HSQC, HMBC and processing routines available in VnmrJ 2.2 C / Chempack 4.0 were applied. The probe temperature was maintained at 298 K and a standard 5-mm NMR tube was used. ^{1}H and ^{13}C chemical shifts (δ) are reported in ppm relative to CHD_2OD (δ=3.349 ppm for ^{1}H) and CD_3OD (δ=49.92 ppm for ^{13}C). The Ph. Eur. method was applied as detailed in Table 1 with the following modifications: acetonitrile was removed and 8% v/v sodium phosphate (pH 8.0) was used instead of 5% v/v potassium phosphate buffer (pH 9.0). In order to lock the spectrometer frequency, half of the water content of the eluent was replaced by D_2O. As a result of the high pressure in the LC/NMR system, the flow rate was reduced to 1 mL/min. The temperature of the column was maintained using a water bath with a heating circulator (Lauda E 100, Lauda-Koenigshofen, Germany).

Chemicals and sample preparation for LC-UV

Chemicals

Acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, Netherlands). *tert*-Butanol (99.5%, extra pure), potassium dihydrogen phosphate (KH_2PO_4) and phosphoric acid were acquired from Acros Organics (Geel,

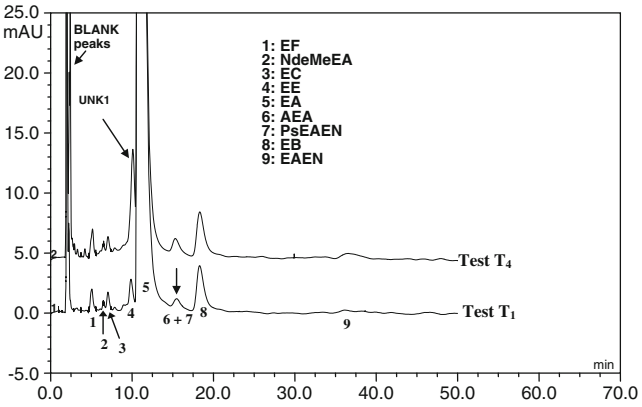


Fig. 3 Overlay of the chromatograms of test solutions at T_1 and T_4 obtained with the Ph. Eur. method

Belgium). *tert*-Butanol was distilled before use. Disodium hydrogen phosphate (Na_2HPO_4) anhydrous was obtained from Fluka (Buchs, Switzerland). Phosphate (0.2 M) buffers pH 7 and 9 were prepared by mixing 0.2 M KH_2PO_4 and 0.2 M Na_2HPO_4.

A mixture, available in our laboratory, containing EA, EB, EC, ED, EE, EF, NdeMeEA, AEA, PsEAHK, PsEAEN, and EAEN was used as a secondary standard for peak identification.

Sample preparation

A freshly prepared 4% m/v sample ES was obtained by dissolving 0.200 g of erythromycin in 5.0 mL of solvent furnished by the manufacturer of the formulation. The solvent contains hydroxypropylcellulose and alcohol. To monitor the degradation of the sample, four test solutions were prepared at different time periods and stored at room temperature. Test T_1 (0 days) was prepared at the time of analysis. Test T_2, Test T_3 and Test T_4 were 14-, 42- and 142-day-old solutions, respectively. The test solutions for analysis were prepared as follows: 4.5 g of sample ES solution was diluted to 20.0 mL

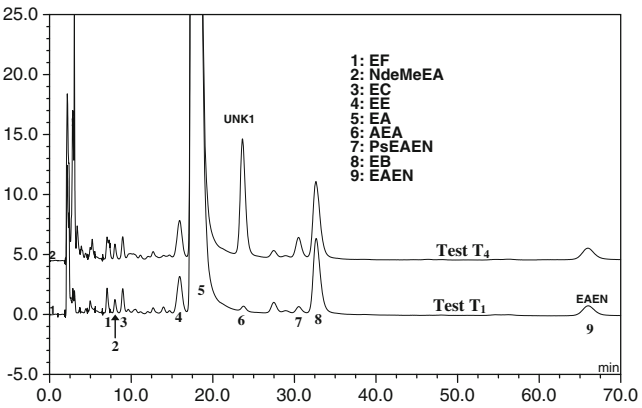


Fig. 4 Overlay of the chromatograms of test solutions at T_1 and T_4 obtained with the Astec method

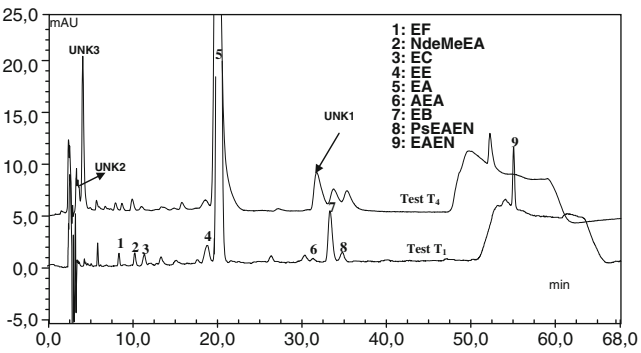


Fig. 5 Overlay of the chromatograms of test solutions at T_1 and T_4 obtained with the XTerra method

with methanol, and 20 μL of this solution was injected into the chromatographic system.

Chemicals and sample preparation for LC/MS

Chemicals

Acetonitrile LC/MS grade was purchased from Biosolve (Valkenswaard, Netherlands). Ammonium acetate was purchased from Acros Organics (Geel, Belgium). *tert*-Butanol, disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained as mentioned in "Chemicals". EA reference substance was obtained from the European Directorate for the Quality of Medicines (Strasbourg, France).

Reference and sample preparation

To obtain optimal intensities, the MS was tuned using EA reference substance dissolved in methanol/water (4:6, v/v)

Table 2 Peak areas (mAU min) measured in the chromatograms

Peak	Test T_1	Test T_2	Test T_3	Test T_4
Ph. Eur. method				
EE+UNK1	0.73	3.54	3.92	4.47
EA	79.87	76.49	72.39	73.94
PsEAEN+AEA	0.62	0.69	0.69	0.97
EAEN	0.21	0.63	0.96	1.03
Astec method				
EA	126.00	118.90	115.32	115.25
AEA+UNK1	0.45	5.10	5.96	6.32
PsEAEN	0.35	0.75	0.96	1.19
EAEN	1.25	1.37	1.50	1.46
XTerra method				
EA	95.52	91.80	88.17	89.06
AEA+UNK1	0.75	4.20	4.68	5.51
PsEAEN	0.34	0.55	0.62	0.98
EAEN	1.33	1.47	1.71	1.77

at a concentration of 0.01 mg/mL. For LC/MS investigation of UNKs, 1 mL of sample ES was diluted to 2 mL using methanol.

Chemicals and sample preparation for NMR and LC/NMR

For off-line NMR measurements 5.0 mg of each reference compound (EA and PsEAHK) was dissolved in 600 μL methanol-d_4 (99.8 atom% D, Sigma-Aldrich, Hungary). *tert*-Butanol (99.91%) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and was distilled before use. Sodium phosphate dibasic dihydrate was supplied by Reanal (Budapest, Hungary), whereas phosphoric acid (85 wt.%) and D_2O (98 atom% D) were obtained from Fluka (Budapest, Hungary). The sample preparation for stop-flow LC/NMR was the same as mentioned in "Sample preparation". The injected volumes were 20 and 100 μL respectively.

Results and discussion

LC-UV investigation

Test solutions were analysed with the three different LC-UV methods. Overlays of the chromatograms of test solutions T_1 and T_4 are shown in Figs. 3, 4 and 5. One peak (UNK1) considerably increased from T_1 to T_4. To separate UNK1 from EA in the Ph. Eur. method, a higher concentration of *tert*-butanol (20% instead of 16.5%) was used, but this caused co-elution of UNK1 with EE. In the other two methods, UNK1 co-eluted with AEA. To examine the change in peak area distribution in sample ES, the peak areas were measured at the different time periods. It was difficult to attribute the peaks in the first part of the chromatograms obtained with the three methods. For this reason, only the labeled peaks in Figs. 3, 4 and 5 were considered. Results for these peaks, the areas of which did

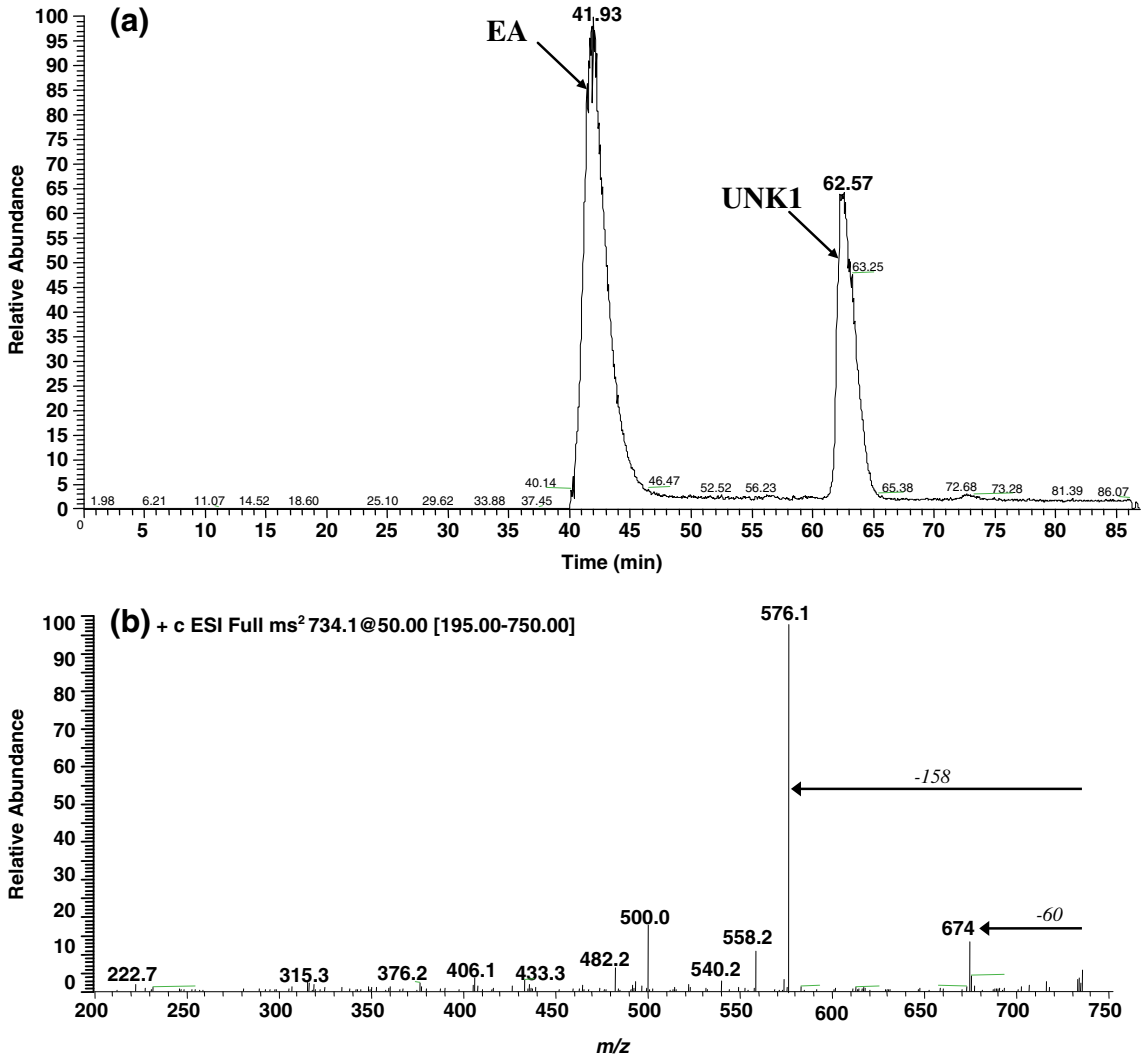


Fig. 6 **a** Typical total ion chromatogram of the top fraction of UNK1. **b** $[M+H]^+$ collision-induced dissociation (CID) spectra acquired for UNK1 ($[M+H]^+$ *m/z* 734); the result of isolation and collisional activation of the precursor ions in the ion trap at 50% collision energy level

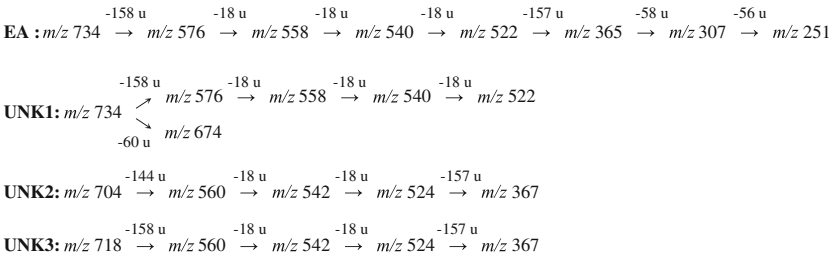


Fig. 7 Schematic representation of the fragmentation pathways for EA, UNK1, UNK2 and UNK3

change significantly, are summarized in Table 2. The data obtained with the Ph. Eur. method showed that the areas of EF, NdeMeEA, EC and EB were constant over time, whereas the area of UNK1 (RRT=0.91), which co-eluted with EE, increased strongly. The areas of PsEAEN+AEA and EAEN grew slightly, whereas the EA area decreased as expected. The results of the analysis with the Astec method indicated that here the area of EE remained stable during the period studied, whereas the area of AEA increased strongly and the areas of the other peaks behaved as discussed for the Ph. Eur. method. The data obtained with the XTerra method confirmed those obtained with the Astec method. So, it was concluded that the compound formed upon storage (UNK1) is neither EE nor AEA, but an unknown substance which co-eluted with EE in the Ph. Eur. method and co-eluted with AEA in the other two. On the basis of the LC-UV results and information from the literature [25], it might be supposed that the decomposition product which co-eluted with AEA corresponds to PsEAHK. However, PsEAHK was not reported to be separated in front of EA in the Ph. Eur. method, nor was it reported as being formed in formulations in such important quantities (up to 5%).

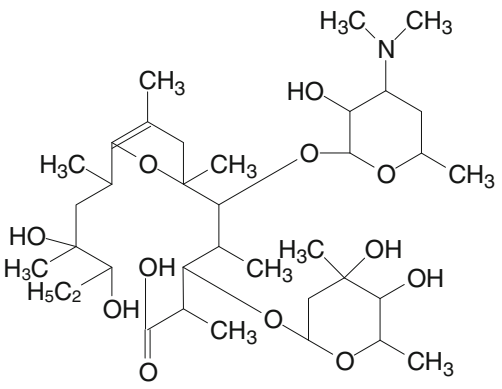
Moreover, two additional peaks at 3.4 min (UNK2) and 3.9 min (UNK3) were observed in the modified XTerra method [30] because of its higher selectivity compared with the other methods in which they were not well separated from the blank peaks. The relative areas of the peaks at 3.4 min and 3.9 min at T_4 were 1.4% and 7.0% respectively. These peaks were not observed at T_1. A detailed description of the separation and structural elucidation of all these impurities is given in the following sections.

LC/MS investigation of the unknowns

Tuning and MS investigation of the reference substances were done according to the procedure mentioned by Pendela et al. [29]. For the investigation of UNK1 by MS, a volatile mobile phase was used. However, because of poor selectivity between the impurities, it was not possible to locate UNK1 in the chromatogram obtained with the volatile mobile phase. Therefore, using a valve-switching system, the UNK1 was collected as a fraction from the Ph. Eur. system and injected into the volatile one. In this way, the sample was desalted before it was introduced into the MS.

As UNK1 was not completely separated from the main EA component in the Ph. Eur. method, the full MS spectrum of the collected fraction shows two peaks with the same $[M+H]^+$, *m/z* 734 (Fig. 6a). The first peak corresponds to EA. The MS^2 spectrum of UNK1 is shown in Fig. 6b. It should be noted that in the ion trap, UNK1 behaved differently compared with EA and other related substances. In the MS^2 spectrum, losses of 158 u (cladinose sugar) and 60 u were observed. A loss of 60 u has not been observed for EA and related substances with *m/z* 734 [25–29]. A loss of 60 u can be attributed to the loss of propanol (instead of propionaldehyde (−58 u)) at the C-13 position, as would be the case in PsEAHK. It is also remarkable to note that a considerable loss of sensitivity in the ion trap was observed for UNK1. Further investigation of UNK1 by NMR is described in "LC/NMR investigation of UNK1".

Next, the structure of the compound indicated as UNK1 in the XTerra method was further examined, as well as UNK2 and UNK3. As described above for the Ph. Eur. method, UNK1, UNK2 and UNK3 corresponding to the peaks in Fig. 5 were trapped, desalted and investigated using MS. A schematic representation of the fragmentation pathways is given in Fig. 7. The fragmentation pattern of UNK1 in the XTerra method was similar to that of UNK1 in the Ph. Eur method. A detailed description about the



UNK2: ECEN carboxylic acid

Fig. 8 Proposed structure for UNK2

investigation of UNK2 and UNK3 is given below. Since UNK2 was deduced from UNK3, UNK3 is described first. Additional MS spectra are available online as Electronic Supplementary Material.

MS/MS and MS^3 investigation of UNK3 (*m/z* 718) revealed that it has the same sugars as erythromycin A, i.e. cladinose (158 u) and desosamine (157 u). However, unlike erythromycin A, it lost only two water molecules instead of

Table 3 ^{13}C and ^{1}H NMR data for erythromycin A (EA) and UNK1 or pseudoerythromycin A hemiketal (PsEAHK) in CD_3OD

Moiety	No.	EA			PsEAHK		
		^{13}C	^{1}H	m, *J* (Hz), int.	^{13}C	^{1}H	m, *J* (Hz), int.
Macrocycle	1	178.5			179.9		
	2	47.3	2.95	m, 1H	49.6	2.72	m, 1H
	3	82.3	4.03	dd, (9.1, 1.1), 1H	83.0	4.02	d, (10.3), 1H
	4	42.0	2.05	m, 1H	42.2	2.31	m, 1H
	5	85.5	3.62	d, (7.5), 1H	88.1	3.59	d, (11.2), 1H
	6	76.3			86.4		
	7	40.7	1.91	m, 1H	43.3	2.38	m, H
			1.65	d, (15.1), 1H		1.54	d, (6.6), 1H
	8	45.1	2.85	m, 1H	39.8	2.38	m, 1H
	9	223.0[a]			109.2[a]		
	10	42.7	3.14[a]	qd, (6.9, 1.4), 1H	40.6	2.08[a]	qd, (6.7, 2.1), 1H
	11	71.0	3.93[a]	d, (1.2), 1H	75.8	5.84[a]	d, (2.1), 1H
	12	77.5			78.7		
	13	79.0	5.19[a]	dd, (11.0, 2.2), 1H	78.3	3.15[a]	dd, (10.4, 1.2), 1H
	2-CH_3	17.2	1.24	d, (7.1), 3H	15.3	1.32	d, (6.9), 3H
	4-CH_3	11.0	1.16	d, (7.3), 3H	12.2	1.14	d, (7.0), 3H
	6-CH_3	27.9	1.45	s, 3H	31.5	1.43	s, 3H
	8-CH_3	19.9	1.19	d, (5.8), 3H	13.4	0.95	d, (5.6), 3H
	10-CH_3	13.2	1.19	d, (5.8), 3H	12.0	1.21	d, (6.7), 3H
	12-CH_3	18.3	1.18	s, 3H	18.6	1.18	s, 3H
	13-CH_2	23.4	1.93	m, 1H	24.9	1.78	m, 1H
			1.53	m, 1H		1.37	m, 1H
	13-CH_2-CH_3	12.1	0.90	t, (7.3), 3H	12.9	1.04	t, (7.3), 3H
Cladinose	1	98.9	4.94	d, (5.1), 1H	101.1	4.83	d, (4.5), 1H
	2	37.0	2.48	d, (15.1), 1H	37.4	2.46	d, (15.1), 1H
			1.64	dd, (15.1, 5.0), 1H		1.59	dd, (15.1, 4.7), 1H
	3	75.0			75.0		
	3-CH_3	22.4	1.29	s, 3H	22.4	1.26	s, 3H
	3-OCH_3	50.9	3.38	s, 3H	50.7	3.32	s, 3H
	4	80.2	3.07	d, (9.5), 1H	80.6	3.04	d, (9.4), 1H
	5	67.6	4.21	m, 1H	67.3	4.26	m, 1H
	6	20.1	1.32	d, (6.3), 3H	19.2	1.29	d, (6.4), 3H
Desosamine	1	105.1	4.52	d, (7.2), 1H	107.6	4.20	d, (7.3), 1H
	2	73.8	3.29	dd, (10.5, 7.2), 1H	73.0	3.36	dd, (10.5, 7.3), 1H
	3	66.0	2.74	td, (11.3, 3.8), 1H	66.4	2.66	td, (11.3, 3.8), 1H
	4	32.9	1.77	m, 1H	33.4	1.79	m, 1H
			1.24	m, 1H		1.31	m, 1H
	5	70.1	3.72	m, 1H	71.0	3.56	m, 1H
	6	22.8	1.22	d, (6.1), 3H	22.3	1.24	d, (6.2), 3H
	N-$(CH_3)_2$	41.6	2.37	s, 6H	41.9	2.39	s, 6H

[a] Key parameters for structure elucidation

three. As a result of the polar nature of the degradation product, it is eluted at 3.9 min. On the basis of the MS data and literature information it is proposed that this degradation product is EAEN carboxylic acid. The pathway for the formation of UNK3 and its structure are shown in Fig. 2. In slightly alkaline conditions, EA is decomposed to PsEAEN via PsEAHK. PsEAHK was found to be the main degradation product as the pH of the aqueous solution increased. The lactone bond of EA-6,9-hemiketal appeared to be hydrolysed by the base catalysis of the hydroxide ion to EA-6,9-hemiketal carboxylic acid. The reaction was followed by the re-esterification of the carboxylic acid with the C11-hydroxyl group (rearrangement of the lactone bond from C13 to C11) and an internal dehydration reaction of PsEAHK to form PsEAEN [15, 16]. Further, hydrolysis of the C11-lactone bond of PsEAEN might produce the degradation product EAEN carboxylic acid (UNK3). However, EAEN carboxylic acid might also be formed directly from the internal dehydration of EA-6,9-hemiketal carboxylic acid. The former pathway is more favourable due to the increase of the PsEAHK concentration on storage.

UNK2 is a degradation compound with a corresponding molecular ion peak at *m/z* 704 which is not completely separated from the blank peaks. Further investigation revealed that it shows a fragmentation pattern like that of EC. Similar to EC, loss of mycarose (144 u) and desosamine sugar (157 u) residues was observed. However, it showed only a loss of two water molecules instead of three. It indicates that one oxygen atom is absent in the aglycone similar to UNK3. As described for UNK3, it is possible that this degradation product might be EC enol ether (ECEN) carboxylic acid (Fig. 8). In the literature [13–16], degradation studies were carried out only on EA. No literature describes the formation of pseudoerythromycins from EC. However, it might be possible that EC also undergoes degradation similar to EA to form ECEN carboxylic acid. From the above investigation, it was found that these degradation products were never reported earlier. Further confirmation of the structure of UNK2 and UNK3 by NMR was not possible as not enough material was available.

Pseudoerythromycins show a high UV absorbance response compared with EA [18, 30]. Hence, it is probable that the relative percentage areas of EAEN carboxylic acid and ECEN carboxylic acid are overestimated.

LC/NMR investigation of UNK1

To further prove the structure of UNK1 proposed on the basis of the data obtained by LC/MS, an LC/NMR investigation was carried out on this degradation product using the Ph. Eur. method. The structures of EA, the main component of sample ES, and the hypothesized impurity PsEAHK were elucidated in a separate off-line NMR experiment using conventional 1D and 2D NMR spectra. The complete assignments of ^{1}H and ^{13}C NMR resonances were determined by the following procedure. The methyl group of the ethyl moiety of the macrocycle at position 13 possesses a characteristic, well-resolved triplet in the upfield region of the ^{1}H NMR spectra; hence this signal provided a good entry point into the resonance assignments. ^{1}H–^{1}H connectivities were deduced from the COSY experiment, whereas the members of the distinct spin systems were identified using the TOCSY spectra. In the case of the cladinose moiety the 3H intense singlet resonance of the methoxy group served as an entry point. Similarly to that, the desosamine sugar resonances were assigned with the help of ^{1}H–^{13}C multiple bond correlation data, where the singlet of the dimethylamino group was assigned first. Connectivities between the sugar units and the spin systems of the macrocycle were confirmed by the HMBC and NOESY data. Finally, the full ^{13}C assignment was verified by the HSQC dataset for each compound. Spectra are available online as Electronic Supplementary Material.

On the basis of the obtained data (Table 3), the key parameters for structure elucidation of these related structures can easily be established. The most characteristic signal is the H-11 doublet of PsEAHK with a low *J* coupling of 2.1 Hz (Table 3). As this signal is the most deshielded one in the spectrum it was chosen to prove the presence of the hemiketal structure unequivocally. This signal is in a “clear” spectral region, far enough from any suppressed solvent signal and can not be taken for the possible co-eluting H-13 of EA, because the multiplicity of this signal is a doublet of doublets with *J* couplings of 11.0 and 2.2 Hz respectively. In the case of LC/NMR experiments, the freshly prepared sample and the same solution

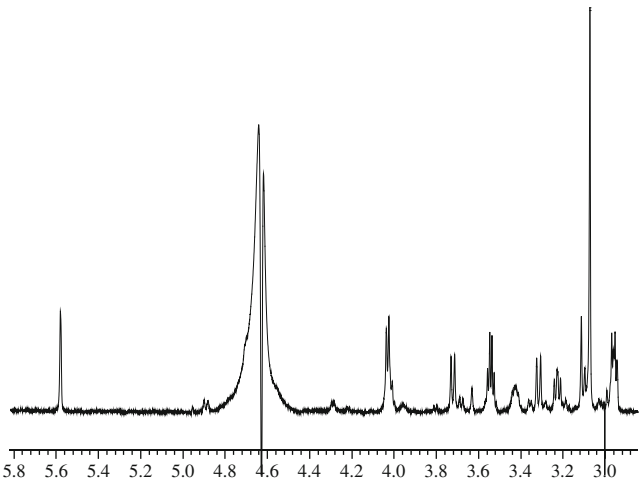


Fig. 9 Downfield part of the stop-flow ^{1}H NMR spectrum of UNK1 with the modified Ph. Eur. method. 1,024 scans were accumulated; the eluent signals were diminished using the WET pulse sequence

after 120 days were monitored. Despite the mentioned modifications to the chromatographic conditions the separation between UNK1 and EA was still reasonable in the latter sample. As shown in Fig. 9, a demonstrative signal in the stop-flow ^{1}H NMR spectrum at 5.58 ppm (J=2.0 Hz) confirms our previous presumption.

Conclusion

Unknown compounds formed in an erythromycin formulation were investigated by LC-UV, LC/MS and LC/NMR. With LC-UV, it was not possible to identify the impurities unambiguously, but using LC/MS and LC/NMR, it was shown that the degradation products corresponded to PsEAHK, EAEN carboxylic acid and ECEN carboxylic acid. EAEN carboxylic acid and ECEN carboxylic acid were newly identified and not reported in the literature earlier.

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