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Investigation of degradation products in a topical gel containing erythromycin and benzoyl peroxide by liquid chromatography–mass spectrometry

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Abstract

Benzamycin®, combining benzoyl peroxide and erythromycin, is a topical gel used in the treatment of acne vulgaris. Because of the reactivity of benzoyl peroxide, preparations containing both erythromycin and benzoyl peroxide might be unstable and degradation products could be formed. To investigate and identify these latter products, a gradient-based liquid chromatographic method using volatile mobile phase constituents was developed. Mass spectrometry data were acquired on solutions containing erythromycin and benzoyl peroxide and on freshly prepared, 2-month-old and 18-month-old samples of Benzamycin®. With the reference spectra as interpretative templates, it was concluded that erythromycin undergoes oxidation, followed by benzoylation.

Keywords: Benzoyl peroxide; Erythromycin; Degradation products; LC–MS

1. Introduction

Common acne, medically known as *acne vulgaris*, is a skin condition characterized by pimples, occurring commonly in teenagers and to a lesser extent in young adults. It is the most common dermatological disorder [1]. Treatment drugs can be oral or topical, and are given as a single drug or as a combination. The combination of the antibiotic erythromycin (Fig. 1) and benzoyl peroxide in a topical gel (Benzamycin®) was reported to have an additive effect compared with the administration of either agent as monotherapy. This combination prevents the emergence of resistant strains of *Propionibacterium acnes* [2]. On the other hand, a stability problem may occur in preparations containing both erythromycin and benzoyl peroxide, because of the reactivity of the latter. It was shown that tretinoin in tretinoin gel microsphere undergoes degradation in the presence of erythromycin-benzoyl peroxide topical gel. The interaction between erythromycin and benzoylperoxide was not studied [3].

The official non-volatile method for the analysis of erythromycin, prescribed by the European Pharmacopoeia [4] and the United States Pharmacopeia [5], is based on work by Paesen et al. [6,7]. Govaerts et al. developed a gradient liquid chromatographic (LC) method on XTerra™ RP_{18}, compatible with mass spectrometry (MS) [8]. Later, Chepkwony et al. developed an isocratic non-volatile method on XTerra™ RP_{18}, which was able to separate more unidentified peaks from the known related substances [9]. Based on the latter method, Chitneni et al. developed a volatile mobile phase and identified eight additional impurities of erythromycin by MS [10]. For the analysis of benzoyl peroxide, the United States Pharmacopeia prescribes an LC method [11]. For the analysis of both active substances, only one method has been published where benzoyl peroxide was assayed by LC, while erythromycin was determined using a microbiological assay [12]. Dehouck et al. developed a non-volatile method showing a good separation and quantitative determination of erythromycin and benzoyl peroxide [13].

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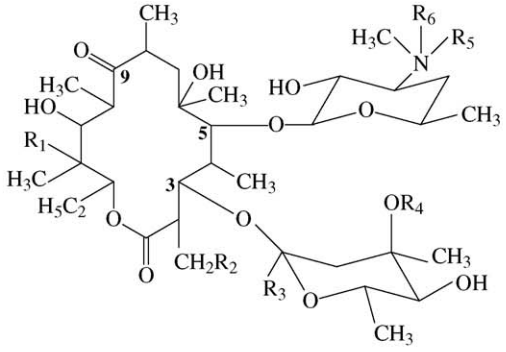
In this study, experiments on solutions containing erythromycin and benzoyl peroxide were carried out before investigating the gels, since reaction products are formed more quickly in solutions than in gels. This would help to identify the reaction products formed in gels. The Benzamycin® samples were stored in a refrigerator, as prescribed, to guarantee the stability during two months. The aim of this study was to investigate the erythromycin derivatives that are formed by the reaction of erythromycin with benzoyl peroxide by LC–MS. A volatile LC method had to be developed.

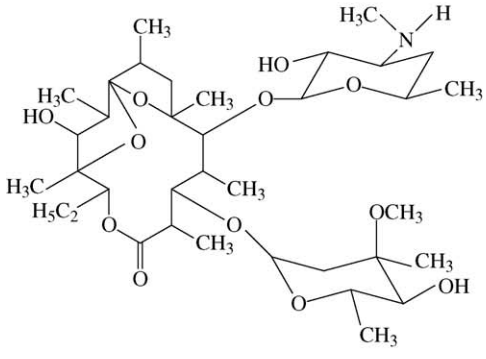
2. Experimental

2.1. Chemicals

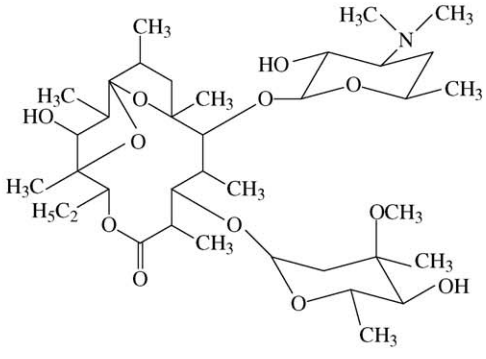
Acetonitrile, HPLC gradient grade and ascorbic acid were purchased from Acros Organics (Geel, Belgium), dipotassium hydrogen phosphate and phosphoric acid were from Merck (Darmstadt, Germany), 2-propanol Chromasolv® and ammonium hydrogen carbonate from Riedel-de Haën (Seelze, Germany) and glacial acetic acid p.a. from VWR (Leuven, Bel-



	m/z $[M+H]^+$	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A	734.2	OH	H	H	CH_3	CH_3	-
Erythromycin B	718.2	H	H	H	CH_3	CH_3	-
Erythromycin C	720.1	OH	H	H	H	CH_3	-
Erythromycin D	704.1	H	H	H	H	CH_3	-
Erythromycin E	748.0	OH	-O-		CH_3	CH_3	-
Erythromycin F	750.2	OH	OH	H	CH_3	CH_3	-
N-demethylerythromycin A	720.1	OH	H	H	CH_3	H	-
Erythromycin A N-oxide	750.2	OH	H	H	CH_3	CH_3	O
Erythromycin C N-oxide	736.2	OH	H	H	H	CH_3	O
Erythromycin E N-oxide	764.3	OH	-O-		CH_3	CH_3	O
Erythromycin F N-oxide	766.2	OH	OH	H	CH_3	CH_3	O

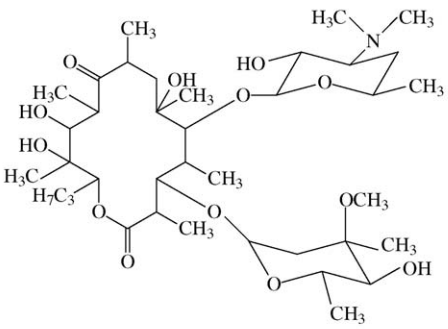


Anhydro-N-demethylerythromycin A

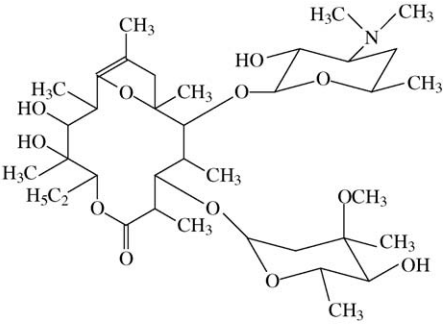


Anhydroerythromycin A (AEA)

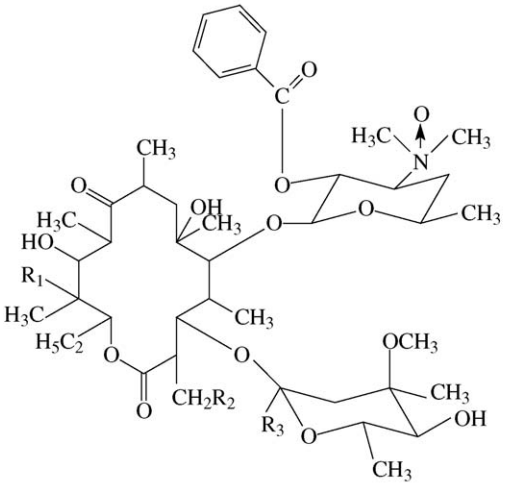
Fig. 1. Chemical structure of erythromycin A, its related substances and proposed structures for the related substances formed by the reaction with benzoyl peroxide.



13-propyl-13-desethyl-erythromycin A



Erythromycin A enol ether (EAEN)



	m/z $[M+H]^+$	R_1	R_2	R_3
Monobenzoyl EA N-oxide	854.3	OH	H	H
Monobenzoyl EB N-oxide	838.2	H	H	H
Monobenzoyl EE N-oxide	868.1	OH	-O-	
Monobenzoyl EF N-oxide	870.2	OH	OH	H

Fig. 1. (*Continued*).

gium). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify glass-distilled water.

2.2. Samples

A pure reference substance of erythromycin A (EA), obtained by open-column chromatography followed by subsequent crystallization and reference substances of erythromycin E (EE) [14], erythromycin F (EF) [15], erythromycin A N-oxide (EANO) [16], *N*-demethylerythromycin A (NdMeEA) [17], pseudoerythromycin enol ether (PsEAEN) [18,19], pseudoerythromycin A hemiketal (PsEAHK) [18], anhydroerythromycin A (AEA) [19] and erythromycin A enol ether (EAEN) [20], which were prepared according to procedures found in literature, were available in the laboratory. Reference substances of erythromycin B (EB) and erythromycin C (EC) were obtained from the European Pharmacopoeia (Strasbourg, France). An impure sample, known to contain erythromycin D (ED) and mainly EA, as verified by thin-layer chromatography, was also available [21]. Several structures are shown in Fig. 1. Erythromycin was from Abbott laboratories (North Chicago, Illinois, USA) and benzoyl peroxide from Federa (Brussels, Belgium). Samples (Benzamycin®, Trenker, Brussels, Belgium) contained 3% m/m of erythromycin and 5% m/m of benzoyl peroxide. Because of stability reasons, the company prescribes that, immediately before delivery, the pharmacist should dissolve the erythromycin (in 10% overdose) in ethanol and mix this solution with the gel containing all other components. This preparation is labelled to be stable for 2 months if stored in a refrigerator (2–8 °C).

2.3. Sample preparation

2.3.1. Solution of erythromycin and benzoyl peroxide

A solution containing 3 mg/mL of erythromycin and 1 mg/mL of benzoyl peroxide was prepared in ammonium bicarbonate buffer (pH 7.0; 0.2 M)–acetonitrile (50:50, v/v). 0.2 M acetic acid was used to adjust the pH of the buffer before bringing to volume. The chosen concentrations of active substances correspond to an equimolar mixture, and this implies that the concentration of benzoyl peroxide is lower than that in the gel. For identification purposes, 0.2 mg/mL of EA, EB, EC, EE, EF, NdMeEA, EANO, AEA, benzoic acid and 0.005 mg/mL of PsEAEN or EAEN were dissolved in the above-described solution.

2.3.2. Extraction of erythromycin and related compounds from the Benzamycin® gel

For the extraction of erythromycin, ammonium bicarbonate buffer (pH 7.0; 0.2 M)–acetonitrile (75:25, v/v) was used. 1.000 g of gel was diluted with this mixture, brought to 25.0 mL and stirred for 30 min. The solution was filtered through a 0.2 μm membrane filter and 100 μL of the filtrate was analyzed by LC. A higher concentration of acetonitrile was not used, since this would extract more benzoyl peroxide.

2.4. LC instrumentation and chromatographic conditions

The LC apparatus consisted of a Spectra SYSTEM P1000XR quaternary pump, a Spectra SERIES AS100 autosampler equipped with a 20 μL loop, a variable wavelength Spectra 100 UV–vis detector set at 215 nm, all from Thermo Separation Products (Fremont, CA, USA) and connected to a computer with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK). Mobile phases were degassed before use by sparging helium. The LC system was coupled to a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionisation (ESI) source operated in the positive ion mode. Data were recorded and processed with Xcalibur software (Thermo Finnigan). As a stationary phase, an XTerra™ RP_{18} 5 μm column (250 mm × 2.1 mm i.d.) was used. The column was kept at 55 °C in a water bath with a heating immersion circulator (Julabo, Seelbach, Germany).

The EA reference substance was used to tune the instrument. This tune file was used during the subsequent investigation of erythromycin fractions. Nitrogen supplied by a Nitroprime TM Membrane unit, type SNIFF (AGA, Lidingö, Sweden) was used as auxiliary and sheath gas. Helium was used as collision gas in the ion trap. For MS/MS investigation, the protonated EA was isolated in the ion trap and collisionally activated with different collision energy levels (CEL) to find the optimal CEL (28%), which generated the highest intensity of product ions needed for further collision-induced dissociation (CID) experiments. CID spectral data or LC/MS^3 spectra were obtained for the product ions relevant to the identification of the unknowns.

3. Results and discussion

3.1. Development of a volatile LC method compatible with MS

A problem in combining LC and MS is the incompatibility of non-volatile mobile phase additives and MS detection. Until now, LC of Benzamycin® has been performed with mobile phases containing non-volatile additives and using column dimensions of 250 mm × 4.6 mm i.d. [13]. Here, a mobile phase with a volatile buffer was developed using an XTerra™ RP_{18} 5 μm column (250 mm × 4.6 mm i.d.). Among the tested volatile buffers, ammonium acetate and ammonium bicarbonate, the latter gave a better separation, although the overall selectivity was less good in comparison with the phosphate buffer used in references [10,13]. The final mobile phase consisted of 2-propanol–acetonitrile–ammonium bicarbonate buffer 0.2 M (adjusted to pH 7.0 using 0.2 M acetic acid before bringing to volume)–water (18.75:6.25:5:70, v/v/v/v).

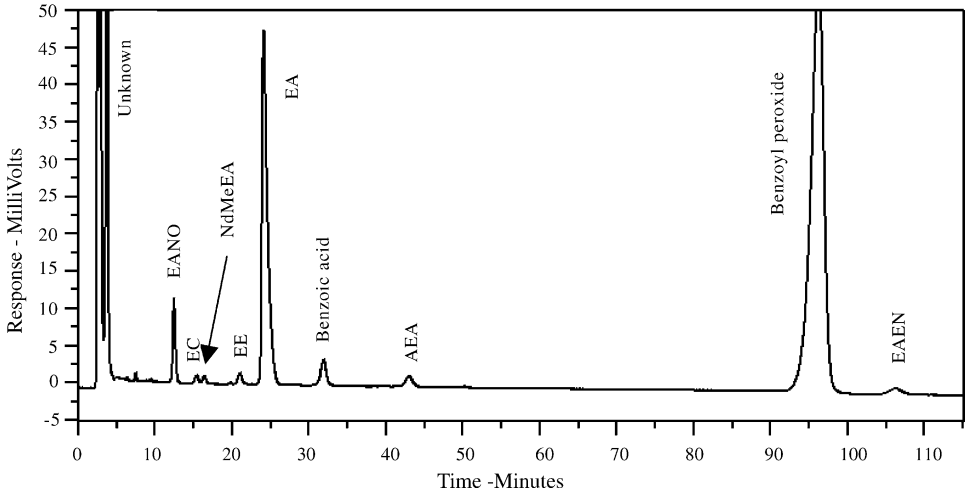


Fig. 2. Typical chromatogram of an erythromycin-benzoyl peroxide solution immediately after preparation of the mixture. Stationary phase: XTerra™ RP_{18} 5 μm (250 mm × 4.6 mm i.d.) at 55° C. Mobile phase: 2-propanol–acetonitrile–ammonium bicarbonate (pH 7.0; 0.2 M)–water (18.75:6.25:5:70, v/v/v/v), Flow rate: 1 mL/min, sample: erythromycin (3 mg/mL)-benzoyl peroxide (1 mg/mL), injection volume: 100 μL, detection: UV at 215 nm. See Fig. 1 for compound abbreviations.

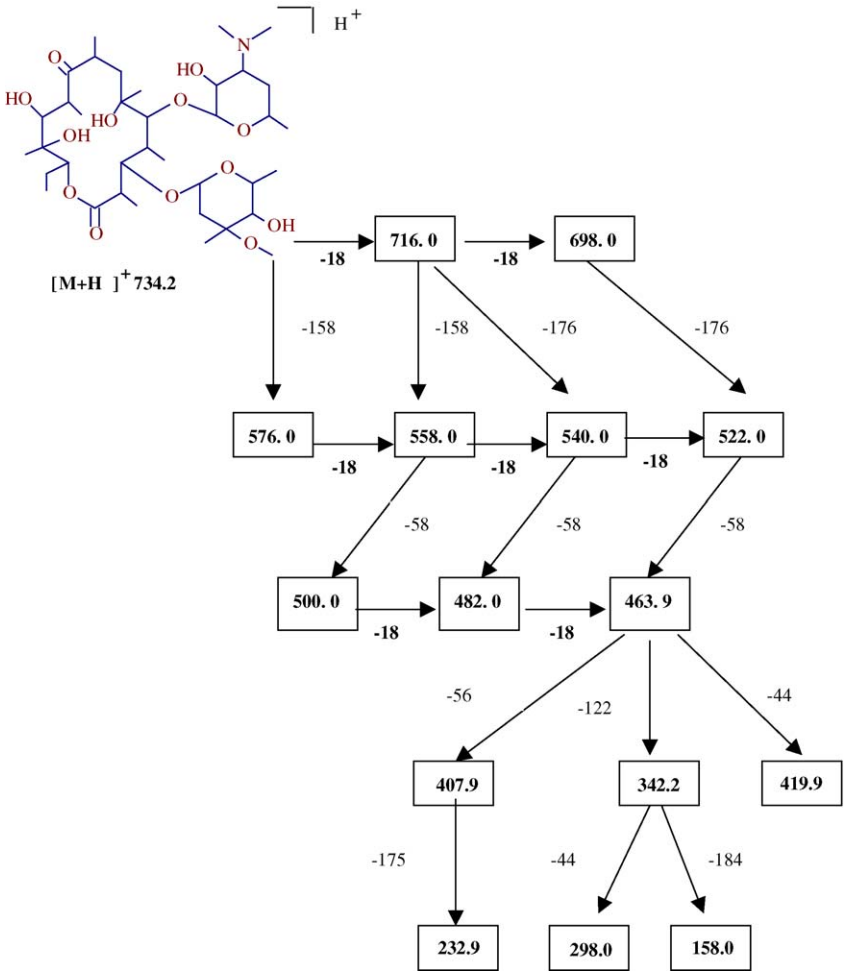


Fig. 3. Fragmentation scheme for erythromycin A based on the MS^2, MS^3 and MS^4 experiments.

As shown in Fig. 2, all erythromycin related compounds like EA, EANO, EC, EE, AEA and NdMeEA, were well separated using a flow rate of 1 mL/min. The same mobile phase was also able to separate benzoyl peroxide. In the chromatogram, some fast eluted (polar) compounds were detected as huge peaks, whereas other possible reaction products might be retained on the column (non-polar compounds). Further investigation on solutions required a gradient system. Investigation of gels was carried out using a column with a smaller diameter, XTerra^{TM} RP_{18} 5 μm (250 mm × 2.1 mm i.d.) and a flow rate of 0.2 mL/min to facilitate the hyphenation with the MS detector.

3.2. Gradient method

Two mobile phases were used: 2-propanol–acetonitrile–ammonium bicarbonate buffer (pH 7.0; 0.2 M)–water, A (18.75:6.25:5:70, v/v/v/v) and B (18.75:40:5:36.25, v/v/v/v). Acetic acid (0.2 M) was used to adjust the pH of the buffer to pH 7.0 before bringing to volume. A three-step gradient was applied: 100% of mobile phase A (0–40 min), linear increase to 100% of mobile phase B (40–60 min) and 100% of mobile phase B (60–90 min).

3.3. MS of the reference substances

The fragmentation behavior of the erythromycins was studied using the reference substances. The fragmentation of the main compound EA $[M+H]^+$ at *m/z* 734.2 is summarized in Fig. 3. Loss of the cladinose residue (158 u) and two waters leads to the ion at *m/z* 540.0. This ion fragments further to *m/z* 463.9 by loss of water and loss of the starter acid unit of the polyketide ring under the form of a propionaldehyde (58 u). The loss of 2,6-dimethylphenol (122 u) leads to *m/z* 342.2. CID of the ion at *m/z* 342.2 leads to *m/z* 298.0 by neutral loss of 44 u, consistent with the loss of CO_2. Loss of 184 u from *m/z* 342.2 yields a desosamine ion at *m/z* 158.0.

Important information was obtained from the fragmentation of *m/z* 522.0. After isolation and collisional activation, the ion at *m/z* 522.0 dissociates predominantly to the ions at *m/z* 463.9, 407.9, 342.2, 232.9 and 158.0. The consecutive losses of 58 and 56 u to, respectively, 463.9 and 407.9 were described by Gates and co-workers [22]. Their deuterated experiments with high resolution Fourier Transform Ion Cyclotron Resonance apparatus showed that the first loss of 58 u is due to loss of the starter acid unit of the polyketide ring under the form of a propionaldehyde (Fig. 4) and the second loss of 56 u

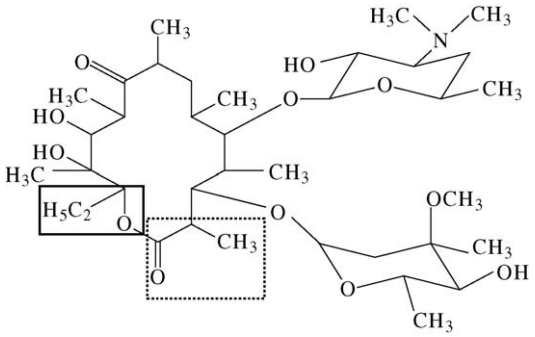


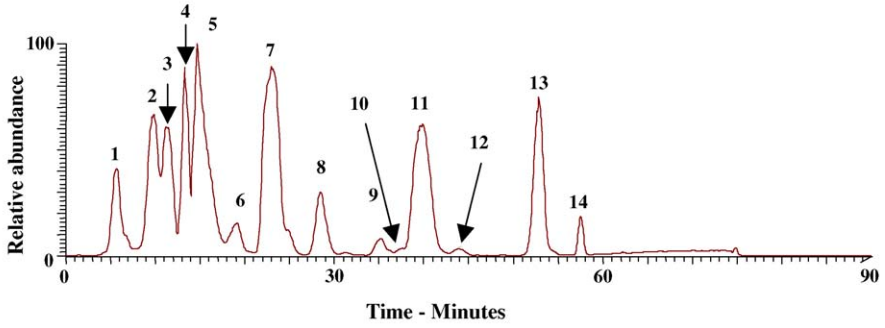
Fig. 4. According to described biosynthetic routes, the aglycone of erythromycin is formed by incorporation of seven propionate units, one of which is used as the starter acid. The starter acid of EA is boxed with a full line. The neighboring oxygen containing unit is boxed with a dotted line.

involves the neighboring oxygen-containing unit of the ring. All abundant product ions observed in the second-generation product ion scan were further investigated in different MS^4 experiments.

Mass spectra of EB, EC, ED, EE, EF, NdMeEA, EANO, PsEAEN, PsEAHK, AEA and EAEN were similarly recorded. The mass spectrometric data were incorporated in a library with as main objective the confirmation of their presence in a commercial sample and the use as interpretative templates for the structure elucidation of unknowns [10].

3.4. MS of the solutions containing erythromycin and benzoyl peroxide

Experiments on erythromycin-benzoyl peroxide solutions (Fig. 5) were carried out before investigating the gels, since reaction products are formed more quickly in solutions than in gels. Fig. 2, showing the chromatogram of an erythromycin-benzoyl peroxide solution immediately after the preparation, only reveals oxidized products but no benzoylated products. Fig. 5, however, shows benzoylated oxidized products appearing in the chromatogram obtained with the solution standing for 20 h. This indicates that oxidation of erythromycin is followed by benzoylation with increase in time. Fig. 5 shows the presence



Peak no	*m/z*	Name
1	766.2	**EFNO**
2	736.2	**ECNO**
3	764.3	EENO
4	750.2	EANO
5	720.1	EC
6	720.1	NdMeEA
7	734.2	EA
8	704.1	ED
9	868.1	**Monobenzoyl EENO**
10	748.2	13-propyl-13-desethyl EA
11	854.3	**Monobenzoyl EANO**
12	718.2	EB
13	732.2	**Probably EA epoxide**
14	838.2	**Monobenzoyl EBNO**

Fig. 5. Typical base peak chromatogram of erythromycin and benzoyl peroxide in solution after 20 h with ESI in positive ion mode. Chromatographic conditions as in Fig. 2, with exception of the mobile phase; 2-propanol–acetonitrile–ammonium bicarbonate buffer (pH 7.0, 0.2 M)–water, A (18.75:6.25:5:70, v/v/v/v) and B (18.75:40:5:36.25, v/v/v/v). A three-step gradient elution was performed: 0–40 min, 100% of A; 40–60 min, a linear gradient to 100% of B; 60–90 min, 100% of B. Newly formed and characterized compounds are indicated in bold.

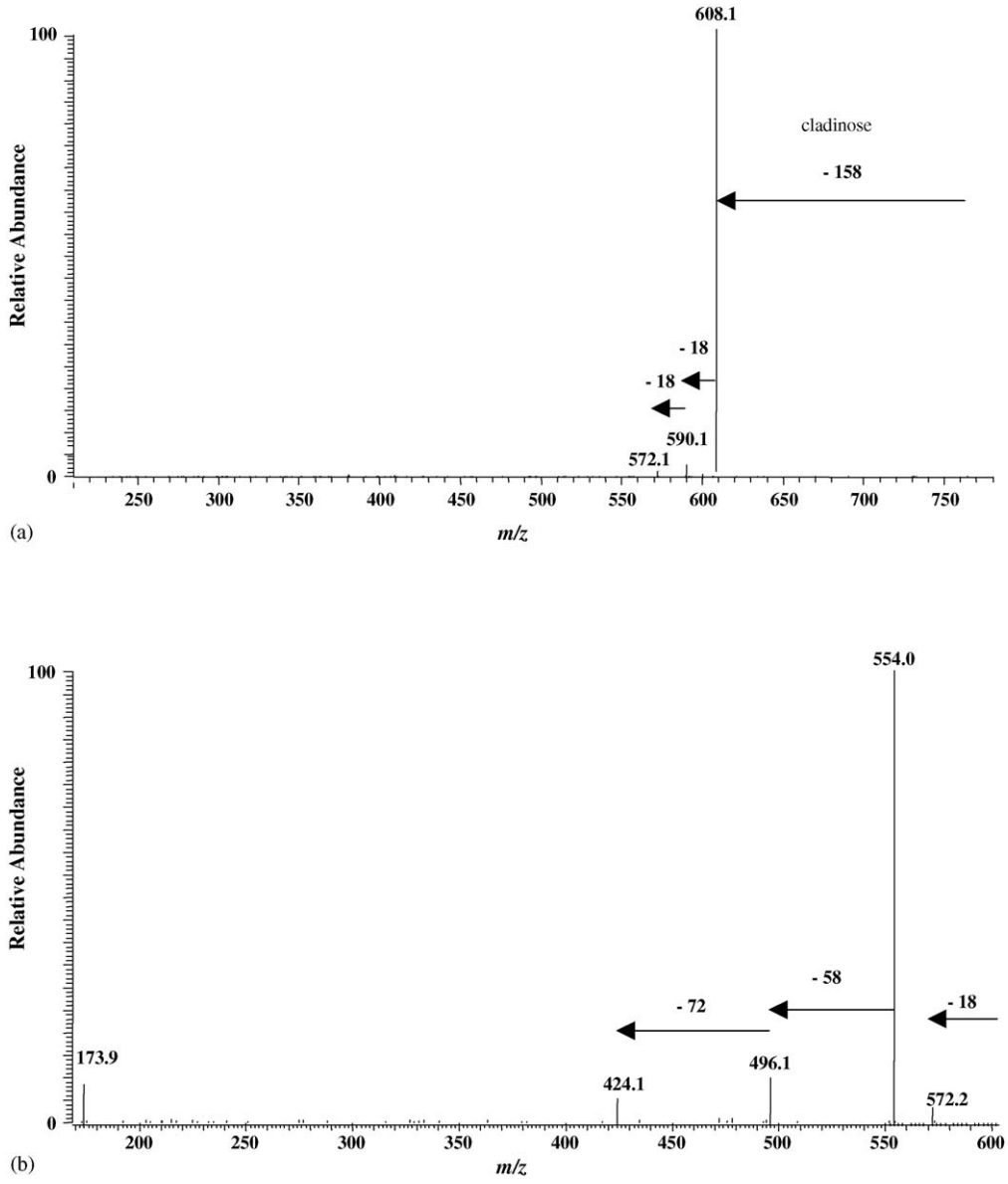


Fig. 6. $[M+H]^+$ CID spectrum (a); and second-generation product ion spectrum (b) acquired for EFNO, the result of isolation and collisional activation in the ion trap at 28% CEL of the precursor ion at *m/z* 766.2.

of known peaks EENO with *m/z* 764.3, EANO with *m/z* 750.2, EC with *m/z* 720.1, NdMeEA with *m/z* 720.1, EA with *m/z* 734.2, ED with *m/z* 704.1, 13-propyl-13-desethyl EA with *m/z* 748.2 and EB with *m/z* 718.2, corresponding with the data provided by Govaerts et al. [8]. The peaks with *m/z* 766.2, 736.2, 868.1, 854.3, 838.2 and 732.2 will be discussed in the next sections.

3.4.1. Mass spectra of the components at m/z 766.2 and 736.2

The product ion spectrum of the precursor ion $[M+H]^+$ at *m/z* 766.2 was compared with the spectra of the reference substance EF (*m/z* 750.2). The product ions in the product ion scan in Fig. 6 differ from the product ions of EF with 16 u. The protonated product ion molecule fragments in Fig. 6(a) to the product ion at *m/z* 608.1 by loss of 158 u, identified as a cladinose sugar. Two subsequent water losses lead to the formation of the product ions at *m/z* 590.1 and 572.1. Fragmentation of the product ion at *m/z* 572.1 (MS^3 spectrum Fig. 6(b)) resulted in the loss of a water molecule and a subsequent loss of 58 and 72 u, due to the starter acid unit and the neighboring oxygen-containing unit. A fragment was present in the spectrum at *m/z* 173.9 instead of *m/z* 158 in EF. The extra mass of 16 Da confirmed that the desosamine moiety was oxidized and this must probably have taken place on the nitrogen, with the formation of erythromycin F N-oxide (EFNO).

A similar pattern was observed for the component at *m/z* 736.2. The initial loss of 144 u was identified as a mycarose sugar, and fragmentation of the product ion at *m/z* 556.1 resulted in the loss of a water molecule and subsequent loss of 58 and 56 u. The latter are due to the starter acid unit and neighboring unit. The proposed structure is erythromycin C N-oxide (ECNO).

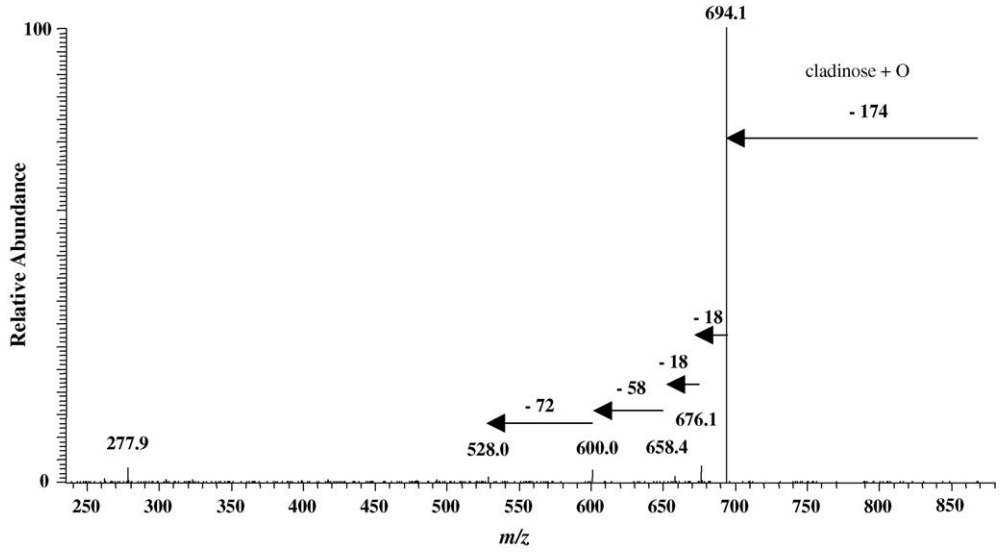


Fig. 7. $[M+H]^+$ CID spectrum acquired for monobenzoyl EENO, the result of isolation and collisional activation in the ion trap at 28% CEL of the precursor ion at *m/z* 868.1.

3.4.2. Mass spectra of the components at m/z 868.1, 854.3 and 838.2

The product ion spectrum of the precursor ion $[M+H]^+$ at *m/z* 868.1 was compared with the spectrum of the reference substance EE with *m/z* 748. There is a difference of 120 Da between the two ions. The protonated molecule fragments to the product ion at *m/z* 694.1 by loss of 174 u, identified as a cladinose sugar with the oxygen of the glycosidic bond (Fig. 7). Two subsequent water losses lead to the formation of product ions at *m/z* 676.1 and 658.4. Further loss of 58 u of the starter acid unit yields an *m/z* of 600.0 and further loss of 72 u yields an *m/z* of 528.0. Instead of the desosamine sugar (*m/z* 158) an ion with *m/z* 277.9 was present. The extra mass of 120 Da, is probably due to oxidation of the nitrogen and benzoylation of the hydroxyl group. The proposed structure is monobenzoyl erythromycin E N-oxide (monobenzoyl EENO).

Similar patterns were obtained for the component at *m/z* 854.3 and *m/z* 838.2, except for the first loss of 158 u, corresponding to a cladinose sugar. Both compounds differ 120 u from their respective reference compounds EA and EB. They have a similar loss of 58 u (starter acid unit) and at *m/z* 278.0 a benzoylated and oxidized desosamine sugar is detected. So, the proposed structures are, respectively, monobenzoyl erythromycin A N-oxide (monobenzoyl EANO) and monobenzoyl erythromycin B N-oxide (monobenzoyl EBNO).

3.4.3. Mass spectra of the component at m/z 732.2

The protonated molecule fragments to the product ion at *m/z* 574.1 by loss of 158 u, identified as a cladinose sugar. Two subsequent water losses lead to the formation of product ions at *m/z* 556.2 and 538.5 (Fig. 8). Since the loss of cladinose was exactly 158 u, the structure elucidation had to be focussed on the remain-

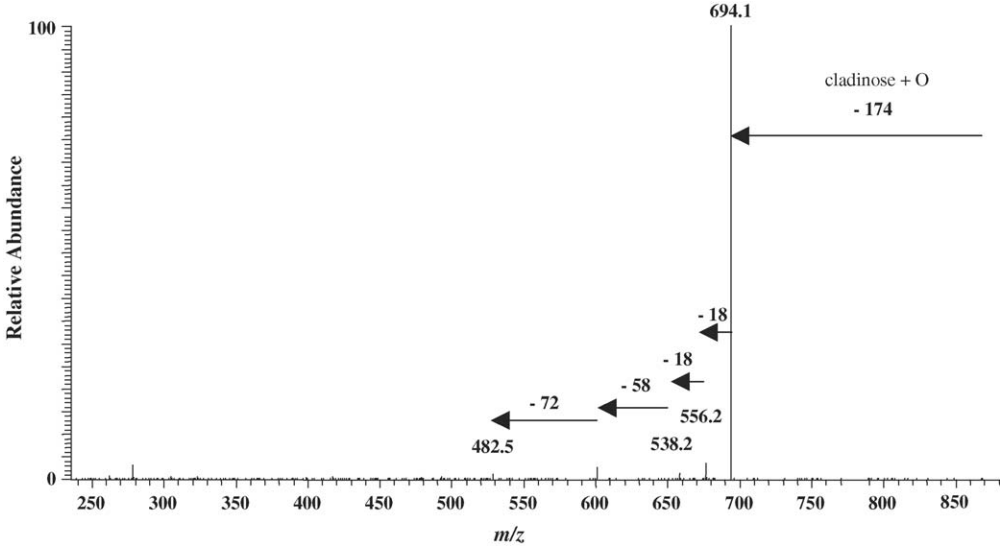
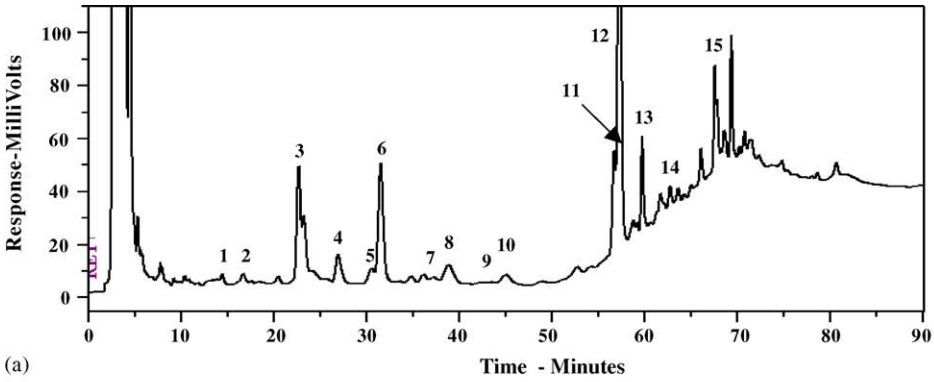
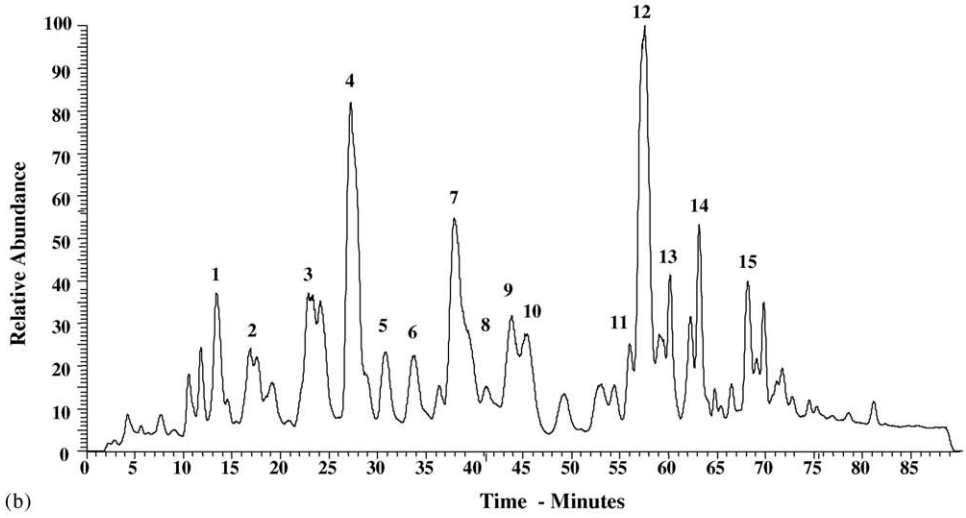


Fig. 8. $[M+H]^+$ CID spectrum acquired for epoxide EA, the result of isolation and collisional activation in the ion trap at 28% CEL of the precursor ion at *m/z* 732.2.





Peak no	*m/z*	Proposed name
1	766.2	**EFNO**
2	748.0	EE
3	750.1	EANO
4	732.2	**Probably EA epoxide**
5	732.2	**Probably EA epoxide**
6	734.2	EA
7	732.1	**Probably EA epoxide**
8	870.2	**Monobenzoyl EFNO**
9	702.0	Anhydro N-demethyl EA
10	854.1	**Monobenzoyl EANO**
11	718.1	EB
12	716.1	AEA
13	838.2	**Monobenzoyl EBNO**
14	716.1	**Probably AEA isomer**
15	716.1	**Probably AEA isomer**

Fig. 9. Typical chromatogram of an 18 month-old Benzamycin® sample. Stationary phase: XTerra™ RP_{18} 5 μm (250 mm × 2.1 mm i.d.), column temperature: 55 °C. Mobile phase, 2-propanol–acetonitrile–ammonium bicarbonate buffer (pH 7.0, 0.2 M)–water, A (18.75:6.25:5:70, v/v/v/v) and B (18.75:40:5:36.25, v/v/v/v). A three-step gradient elution was performed: 0–40 min, 100% of A; 40–60 min, a linear gradient to 100% of B; 60–90 min, 100% of B. Injection volume: 20 μL, flow rate: 0.2 mL/min. Detection (a) UV at 215 nm; and (b) ion trap equipped with ESI source operated in positive ion mode. Newly formed and characterized compounds are indicated in bold.

ing part of the compound. The mass of 732.2 has only 2 units difference with EA (*m/z* of 734.2), hence a radical epoxide formation was considered to be formed from the hydroxyl on C6, C11 or on C2′ in the desosamine moiety. The radical formation would be induced by benzoyl peroxide. The three different potential sites of epoxide formation could explain the appearance of three peaks of *m/z* 732.2 in Fig. 9. The proposed structure is an EA epoxide, involving C6, C11 or C2′ hydroxyl.

3.5. MS of Benzamycin® samples

Full mass spectral acquisition over the mass range *m/z* 155–2000 was performed to gain information about all components in the commercial Benzamycin® samples.

Commercial Benzamycin® samples, freshly prepared, 2-month old and 18-month old were analyzed for the structure elucidation of unknown impurities. Freshly prepared and 2-month-old Benzamycin® samples did not show related substances other than those present in the erythromycin used for the preparation. Analysis of the 18-month-old samples revealed some related substances, which were also present in the erythromycin-benzoyl peroxide solution and also some additional related substances. Fig. 9(a) (LC–UV) and Fig. 9(b) (LC–MS) show chromatograms of 18-month-old commercial Benzamycin® samples.

Mass spectrometric analysis of Benzamycin® samples resulted in the *m/z* values indicated in Fig. 9. The spectra of several peaks matched with the spectra of the reference substances and of impurities found in the erythromycin-benzoyl peroxide solution. The peaks at *m/z* 702.0 and 716.1 were already described by Chitneni et al. [10]. One unidentified peak, having an *m/z* of 870.0 was only observed in the 18-month-old Benzamycin® sample.

3.5.1. Mass spectra of the component at m/z 870.0

The fragmentation spectra of the precursor ion *m/z* 870.0 were compared with the fragmentation spectra of the reference substance EF (*m/z* 750.2). There was a difference of 120 Da between the two ions. Spectral data show that this compound was analogous to EF with a benzoylated and oxidized des-

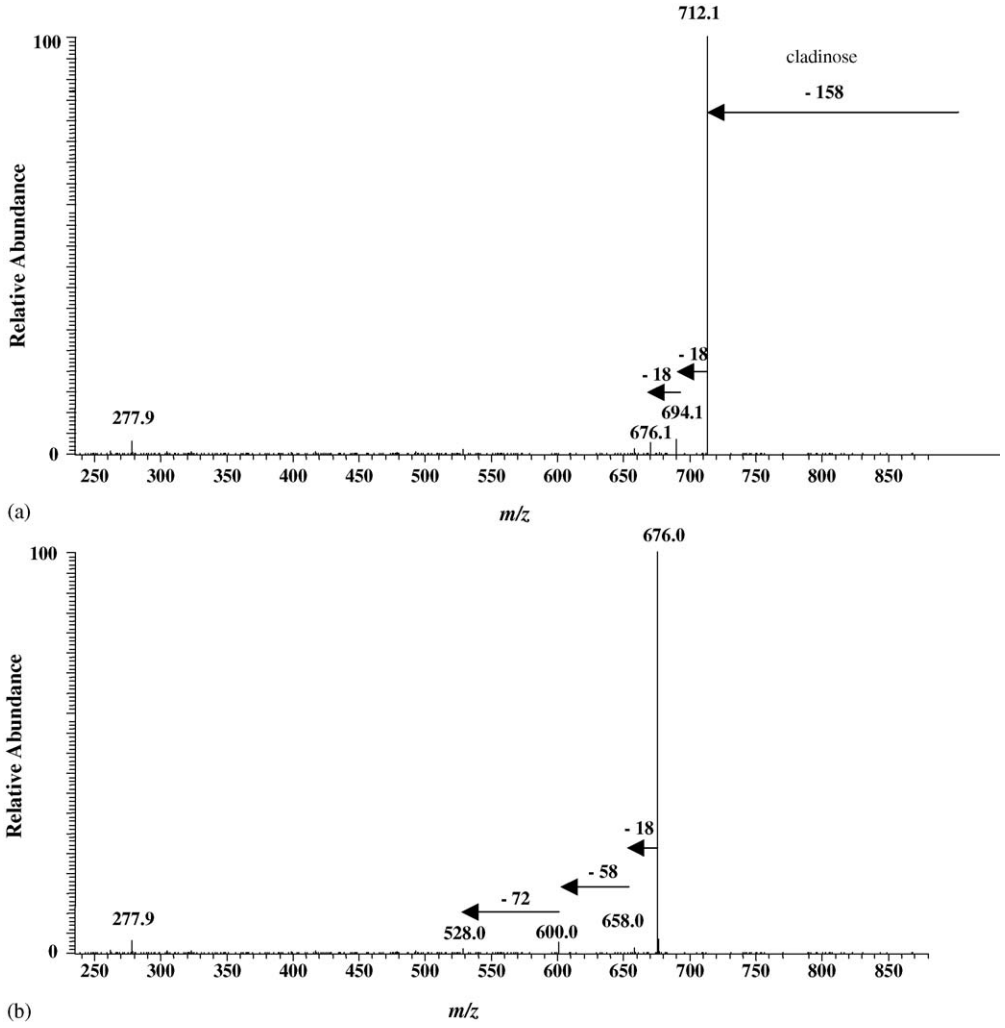


Fig. 10. $[M+H]^+$ CID spectrum (a); and second-generation product ion spectrum (b) acquired for monobenzoyl EFNO, the result of isolation and collisional activation in the ion trap at 28% CEL of the precursor ion at *m/z* 870.0.

osamine. Similar to EFNO, this compound looses first a 158 u cladinose sugar and two subsequent water molecules of 18 u (Fig. 10). With MS^3, a loss of 58 u of the starter acid unit and further loss of 72 u due to the neighboring oxygen-containing unit was observed. In accordance with the compounds with *m/z* 868.1 (monobenzoyl EENO), 854.3 (monobenzoyl EANO) and 838.2 (monobenzoyl EBNO), an *m/z* at 277.9 of the benzoylated and oxidized desosamine sugar was found. The proposed structure is monobenzoyl erythromycin F N-oxide (monobenzoyl EFNO).

3.5.2. Mass spectra of the component at m/z 702.0

Based on the MS/MS and MS^3 spectrum, Chitneni et al. postulated that the compound at *m/z* 702.0 could be anhydro *N*-demethylerythromycin A or *N*-demethyl erythromycin A enolether, but taking the relative retention time in account, the peak was assumed to be anhydro *N*-demethylerythromycin [10].

3.5.3. Mass spectra of the components with m/z 716.1

Fig. 8 shows peak 12 with an *m/z* of 716.1, identified as AEA using a reference compound. Apart from this peak, two additional peaks are observed with the same *m/z*, and the same fragmentation pattern but a different retention time. For both, the proposed structure is an isomer of AEA. Such isomer structures were also reported previously for anhydro *N*-demethylerythromycin A [10].

4. Conclusion

After the development of a gradient-based volatile method for MS, the fragmentation behavior of the unknown erythromycin peaks was studied in comparison with that of the reference substances. The analysis of erythromycin-benzoyl peroxide solutions revealed formation of new erythromycin derivatives, which were identified as EFNO, ECNO, monobenzoyl EENO, monobenzoyl EANO, probably EA epoxide and monobenzoyl EBNO.

It was observed that freshly prepared Benzamycin® and 2-month-old (expiry date) samples showed no additional peaks related to the oxidation or benzoylation of erythromycin. Analysis of 18-month-old Benzamycin® samples revealed formation of EANO, EFNO, probably EA epoxide, probably AEA isomer, monobenzoyl EANO, monobenzoyl EBNO and monobenzoyl EFNO.

From the results it can be concluded that the preparations containing erythromycin and benzoyl peroxide undergo oxidation followed by benzoylation.

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