Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 858–865

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Structural elucidation of the Rifaximin Ph. Eur. Impurity H

Riccardo Stradi [a,*], Donatella Nava [a], Marino Nebuloni [b], Barbara Pastura [a], Elena Pini [a]

[a] DISMAB - Sezione di Chimica Organica "A.Marchesini", Università degli Studi, Milano, Italy
[b] REDOX S.n.c., Monza (Mi), Italy

ARTICLE INFO

Article history:
Received 29 July 2009
Received in revised form 12 October 2009
Accepted 19 October 2009
Available online 28 October 2009

Keywords:
Rifaximin
802 Dalton impurity
European Pharmacopoeia
Structural elucidation
HPLC/MS/MS
Spectroscopic techniques

ABSTRACT

Rifaximin, a semisynthetic, rifamycin-based non-systemic antibiotic is used in the treatment of acute and chronic gastrointestinal disorders. The aim of this study was the elucidation of the molecular structure of the 802 Dalton impurity which was found in Rifaximin industrial batches and reported with an erroneous structure in European Pharmacopoeia 6.5 (2009) [7] monograph as Rifaximin Impurity H. This impurity was isolated from Rifaximin by preparative HPLC and purified by column chromatography. The molecular structure was evidenced by means of ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry and FT-IR.

1. Introduction

Rifaximin is (2*S*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*R*,27*S*,28*E*)-5,6,21,23-tetrahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octamethyl-1,15-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienimino)-benzofuro[4,5-*e*]pyrido[1,2-a]-benzimidazo-25-yl acetate (Fig. 1). Structurally it is characterized by a cyclic structure consisting of an aromatic group and an aliphatic chain ("ansa chain") that forms a bridge between two non-adjacent positions of the aromatic moiety. Rifaximin is a semisynthetic antibiotic not absorbed when administered orally [1]. It inhibits bacterial RNA synthesis, generating an irreversible complex with RNA-polymerase; it is active against Gram-positive and Gram-negative aerobic and anaerobic bacteria [2] and is used in the prevention or treatment of acute and chronic gastrointestinal disorders caused by *Escherichia coli* [3], *Shigella* [4] and *Clostridium difficile* [2]. Rifaximin is synthesized [5] by the reaction between Rifamycin O and 2-amino-4-methylpiridine. Rifamycin O is produced by oxidation of Rifamycin B carried out by *Amycolatopsis mediterranei*, a Gram-positive aerobic bacterium belonging to the order Actinomycetales [6].

The complexity of Rifaximin biosynthetic process gives rise to obtain several impurities probably related to Rifamycin O. The HPLC analysis and the impurities' structures are described in European Pharmacopoeia [7]; the 802 Dalton (MW) impurity structure (Fig. 1) is reported as Rifaximin Impurity H but, to our knowledge, there is no evidence in the literature for its structural elucidation. The aim of this work was to isolate this impurity that was always present in many industrial batches analysed in our laboratory in order to evaluate their analytical profile. The 802 impurity was isolated by preparative HPLC, purified by further chromatographic steps and than structurally characterized using UV, FT-IR, NMR spectroscopies and mass spectrometry.

2. Experimental

2.1. Materials and methods

All reagents and spectroscopic solvents pure grade employed for HPLC and LC/MS analyses and Chloroform-d for NMR were purchased from Aldrich Chemical. The samples of Rifaximin were gentle gift of Interquim SA DE CV (Avenida Antoine Lavoiser, 22, Parque Industrial Cuatitlàn Izcalli – E.do de Mexico).

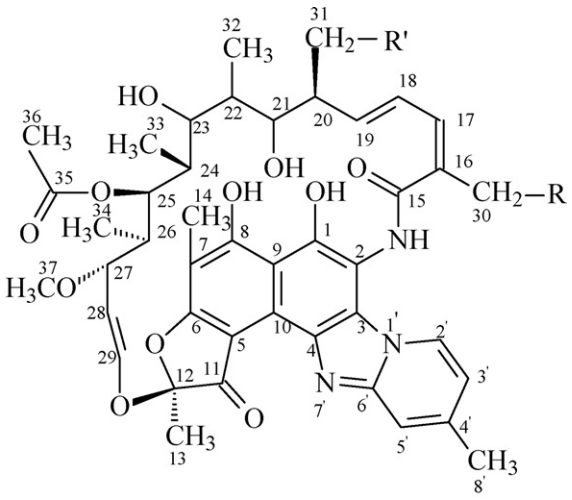
2.2. HPLC

The preparative HPLC system made up of a Waters Delta PREP600E quaternary pump equipped with a manual injector (loop 1 ml), an online preparative degasser and a FCIII Waters fraction collector. The detector was a Diode Array (Waters mod. 2996, MA, USA).

For the isolation of the 802 impurity a Symmetry Shield RP18 (Waters, MA, USA), 150 mm × 19 mm i.d. (5 μm particle size) was employed; acetonitrile–methanol–water (36:32:32, v/v/v) was

* Corresponding author.
E-mail address: riccardo.stradi@unimi.it (R. Stradi).

0731-7085/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.jpba.2009.10.012



R = R' = H Rifaximin
R = H R' = OH Ph. Eur. impurity H
R = OH R' = H 802 Dalton impurity

Fig. 1. Molecular structure of Rifaximin, Rifaximin Impurity H as reported in European Pharmacopoeia and of 802 impurity.

used at a flow rate of 8 ml/min at room temperature. Detector wavelength was set at 276 nm.

2.3. Isolation and purification of 802 impurity

The impurity was isolated from Rifaximin by preparative HPLC, following the experimental condition above described. The sample solution (25 mg/ml) was prepared in mobile phase; 125 separation cycles were carried out. 25.3 mg of crude 802 impurity was obtained which was purified in two steps: (i) five cycles of preparative HPLC were carried out modifying the mobile phase (acetonitrile–methanol–water 30:20:50, v/v/v) in order to obtain a better separation of the peaks; 10 mg of product was obtained and (ii) purification by silica gel column chromatography using chloroform/ethanol (90:10, v/v) as eluent. Almost pure (>95%) 802 impurity (6 mg) was thus obtained.

2.4. UV–vis spectroscopy

UV–vis spectra were scanned by a UV-Vis V-530 Jasco spectrophotometer (Japan), in the 209–600 nm range. Samples (0.017 mg/ml) dissolved in methanol were analysed in 1 ml quartz cuvettes with a 1 cm optical pathway.

2.5. Mass spectrometry

LC/DAD/MS analyses were performed using a Thermo Finnigan (MA, USA) LCQ Advantage system equipped with a quaternary

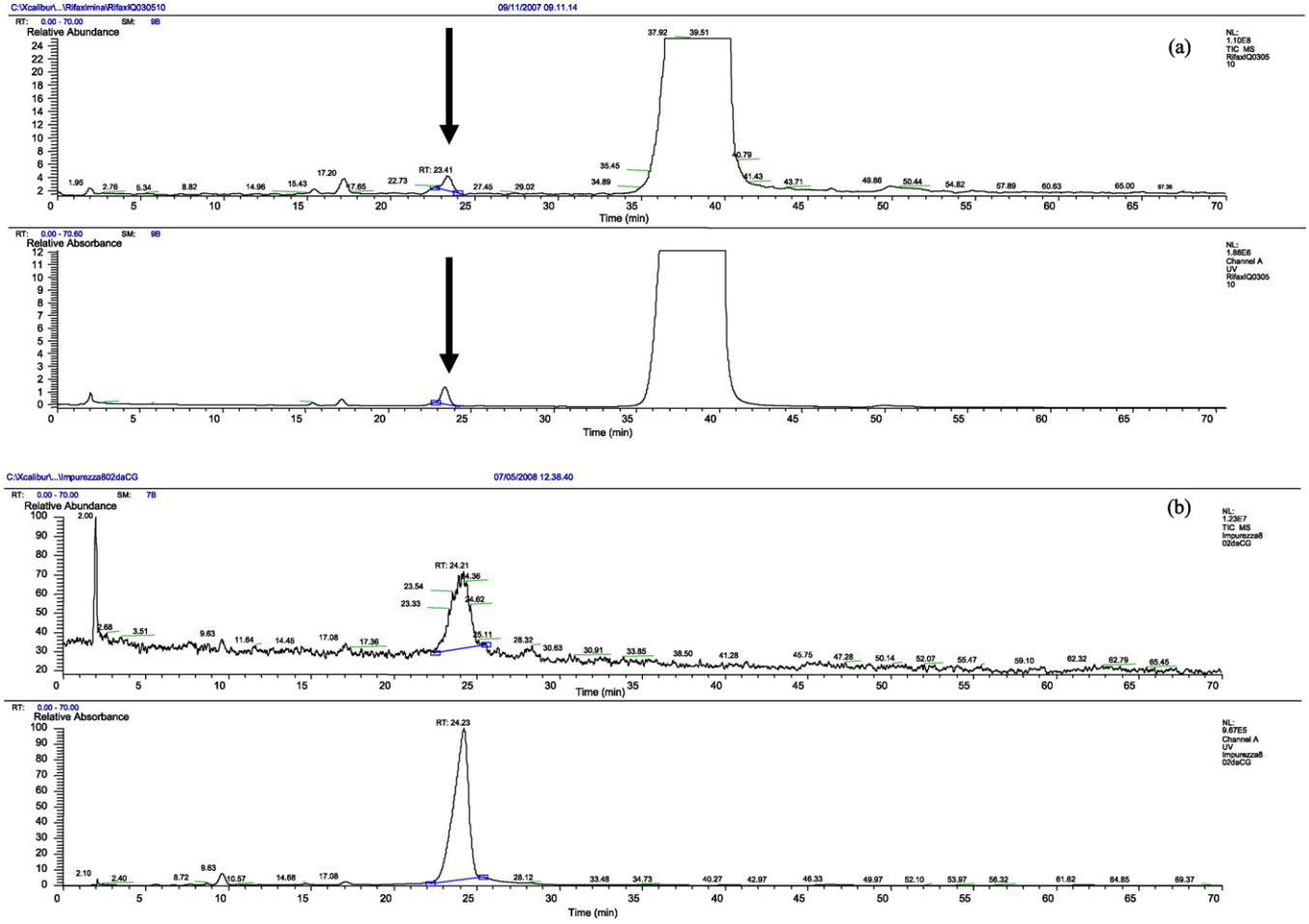


Fig. 2. TIC and PDA chromatograms of Rifaximin (a) and of isolated and purified impurity (b).

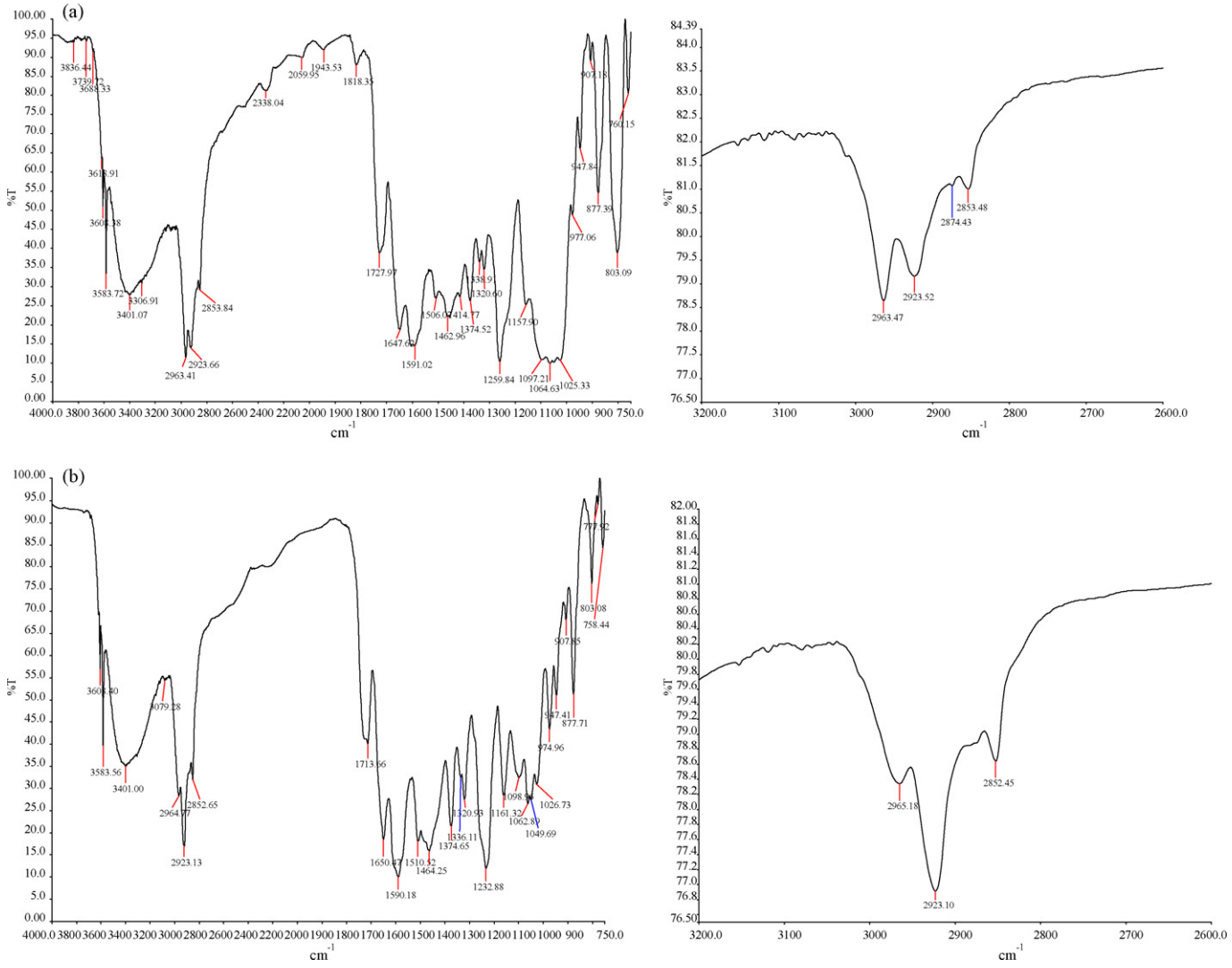


Fig. 3. FT-IR spectra of Rifaximin (a) and 802 impurity (b).

pump, Diode Array Detector (working wavelength 276 nm) and MS spectrometer with an electrospray ionisation source and an "Ion Trap" mass analyser. The LC column employed was a Symmetry Shield RP 18 (Waters, MA, USA), 250 mm × 4.6 mm i.d. (5 μm particle size), mobile phase was acetonitrile–methanol–water (30:20:50, v/v/v) eluted at 0.8 ml/min.

The LC–MS chromatograms and MS spectra were obtained under the following conditions: ionisation, ESI (+/−); source voltage, 4.12 kV; source current, 80.76 μA; sheath gas flow rate, 19.09; capillary temperature, 250 °C; capillary voltage, −27.45 V.

2.6. FT-IR spectroscopy

FT-IR spectra were collected using a Perkin Elmer (MA, USA) FT-IR Spectrometer "Spectrum One" in the 4000 and 750 cm^{-1} spectral region. Samples, diluted with 100 μl of spectroscopy grade chloroform, were analysed by transmittance technique with 32 scansions and 8 cm^{-1} resolution.

2.7. NMR spectroscopy

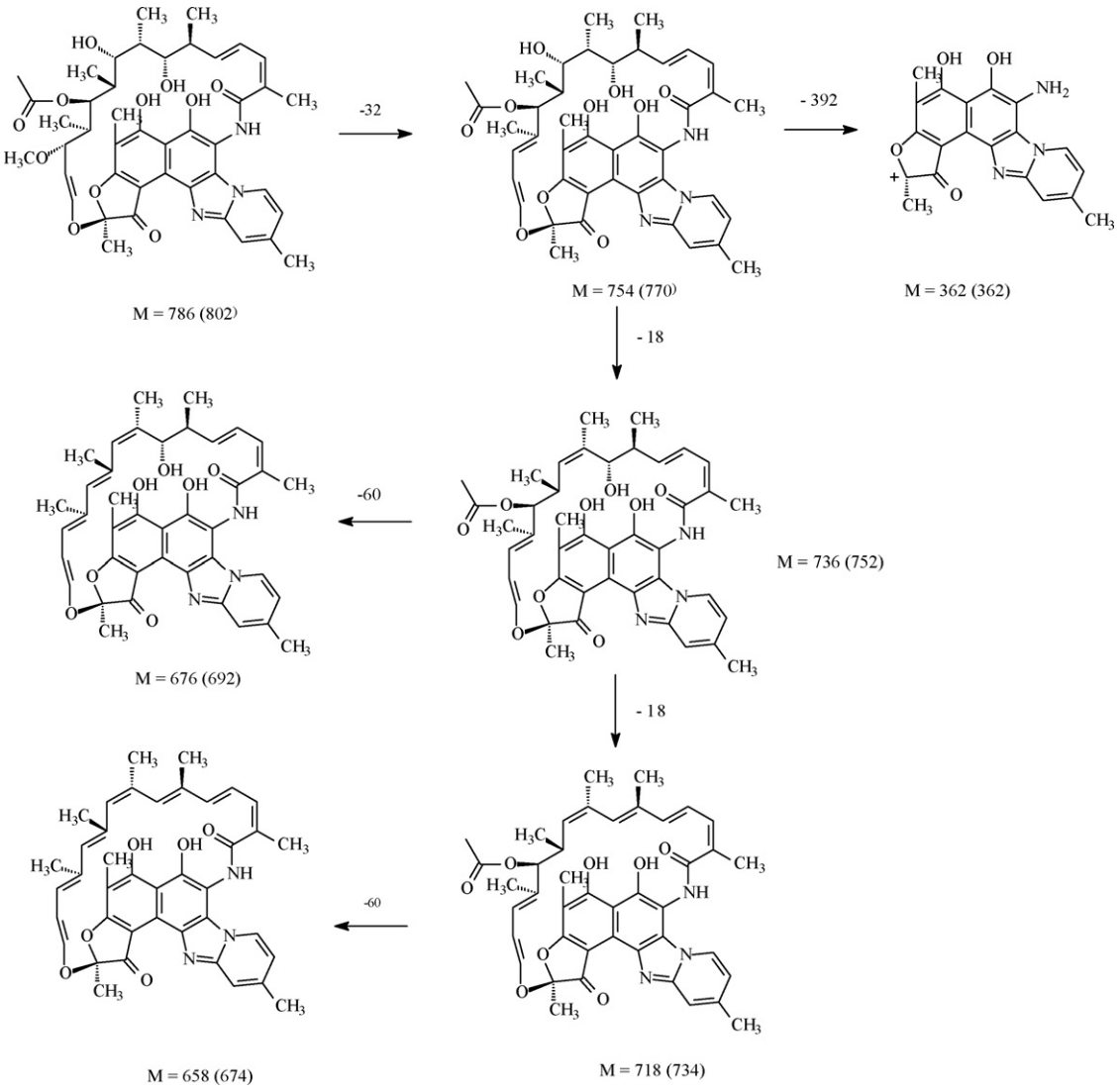
The ^{1}H and ^{13}C NMR and two-dimensional analyses, HMQC and HMBC, were collected by a Bruker Avance 500 (Billerica, MA, USA) operating at 500 MHz. Chemical shifts were expressed as ppm (δ) from TMS. Samples of about 3 mg were dissolved in 0.7 ml of $CDCl_3$.

3. Results and discussion

The Rifaximin industrial batch used for this study shows the HPLC chromatogram reported in Fig. 2a. The chromatographic purity of the isolated and purified impurity (Fig. 2b) was >95%.

3.1. UV–vis spectroscopy

No significant differences were observed by comparing the UV–vis spectra of 802 impurity (λ_{max} 220, 236, 293, 372, 442 nm)



Scheme 1. (+) ESI fragmentation pathway of Rifaximin and 802 impurity. The molecular weights of impurity fragments are reported in brackets.

and Rifaximin (λ_{max} 219, 236, 293, 372, 453 nm). This shows that the aromatic chromophore of the two compounds is the same and therefore the 802 impurity is structurally related to Rifaximin: the difference between the two compounds is located on the "ansa" chain.

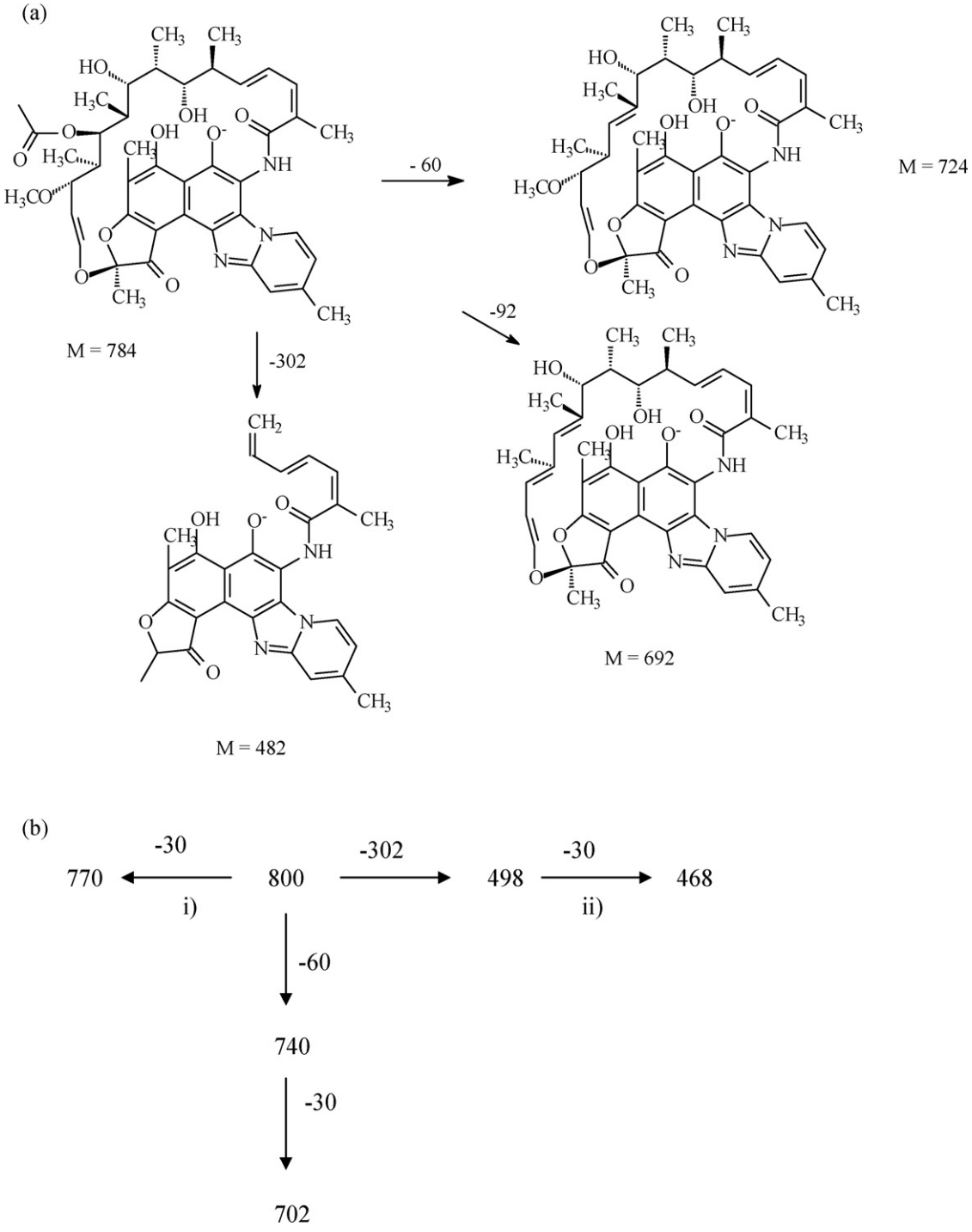
3.2. FT-IR

The infrared spectrum of the 802 impurity showed the main absorption bands at 3608, 3583, 3401, 1727, 1647 cm^{-1} suggesting a strong structural relation with Rifaximin. As shown in Fig. 3, a remarkable and interesting difference was observed in the Csp^3–H stretching zone: the relative intensities of the bands at 2924 cm^{-1} and 2963–2965 cm^{-1} are dramatically different in the two compounds. Actually the increase of the band intensity at 2924 cm^{-1}, characteristic of the CH_2 asymmetric stretching [8], suggested the presence of a CH_2 in the 802 impurity. The fingerprint region showed many differences particularly in the Csp^3–O stretching zone between 1020 and 1100 cm^{-1}.

3.3. Mass spectrometry

The ESI (+) fragmentation pattern of 802 impurity compared to that of Rifaximin (Scheme 1) shows that all the significant fragments possess a MW +16 with respect to the parent compound. Only the 362 fragment is present in the mass spectra of both compounds (Rifaximin and 802 impurity). This experimental evidence confirms that the additional oxygen atom of the impurity is bonded to the "ansa" chain. Taking into account the ESI (−) mass, the spectra are very different from the ESI (+) ones and richer in information. The 802 impurity undergoes a loss of formaldehyde (−30) which takes place in three different fragmentation steps: (i) directly from the ionised molecule; (ii) from the 498 fragment; and (iii) from 740 (M-60) fragment (Scheme 2b). The fragmentation of 498 (482 + O) to 468 is particularly significant because it shows that the oxygen atom is bonded (as CH_2OH) to the C_{30} or C_{31}. The 498 (482 + O) fragment is actually related to the 482 fragment of Rifaximin (Scheme 2a).

From the obtained evidences by ESI (+) and ESI (−) mass spectrometries (Fig. 4) we could conclude that the 802



Scheme 2. (a) (−) ESI fragmentation pathway of Rifaximin. (b) (−) ESI fragmentation pathway of 802 impurity.

impurity structure is 31-hydroxy or 30-hydroxy Rifaximin (Fig. 1).

3.4. NMR spectroscopy

In Table 1 the ^{1}H NMR chemical shifts of Rifaximin [9] and impurity 802 are reported. These data highlight that in Rifaximin spectrum four doublets are present corresponding to CH_3 (31, 32, 33, 34) and six singlets corresponding to CH_3 (8′, 13, 14, 30, 36, 37). The chemical shifts in 802 impurity are approximately the same as those of Rifaximin: all the four CH_3 doublets and five singlets corresponding to CH_3 8′, 13, 14, 36, 37. Moreover an AB system (δ_A = 4.67 ppm; δ_B = 4.73 ppm; J_{AB} = 12.5 Hz) is present in the deuterated spectrum of 802 impurity (Fig. 5). The lack of a CH_3 singlet in comparison with Rifaximin spectrum and the spectroscopic properties of the above mentioned AB system agree with the presence of a = C–CH_2–OH group in the 802 Dalton impurity and disagrees with the structure reported in European Pharmacopoeia for Impurity H. All the other chemical shifts do not significantly differ from those of Rifaximin, confirming that the only difference

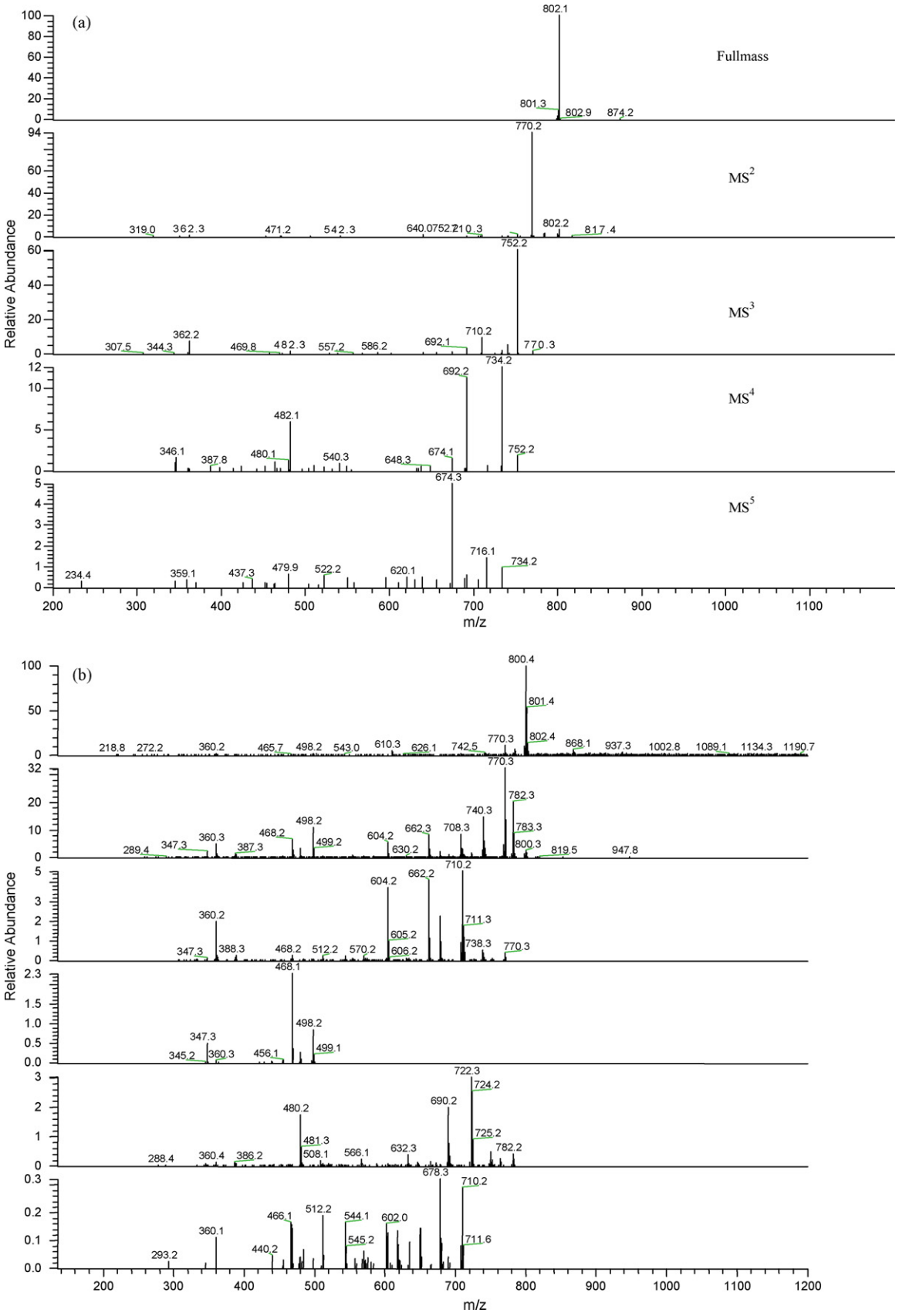


Fig. 4. Direct infusion mass analysis ESI (+) (a) and ESI (−) (b) of 802 impurity.

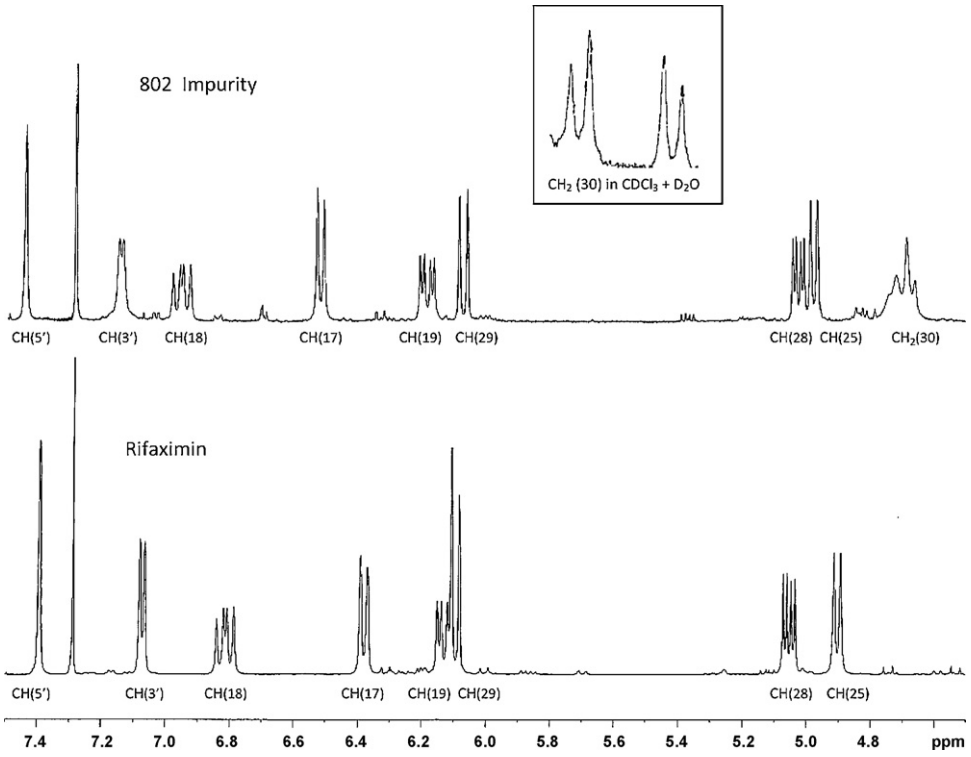


Fig. 5. Partial ^{1}H NMR spectrum (4.6–7.4 ppm) of 802 impurity and Rifaximin. In the box, the, CH_2 (30) signal after deuteration is reported.

between the two molecules is represented in the C_{30} position. Furthermore APT ^{13}C NMR (Fig. 6) of the 802 impurity confirms the absence of an odd peak in the methyl region, while it is clear that the even peak at 64.65 ppm corresponds to the chemical shift of C_{30} in the $=C–CH_2–OH$ group. In the HMQC analysis this peak correlated with the two hydrogens of the AB system. The HMBC analysis showed a correlation between the CH_3 (31) hydrogens and the C (21) and C (20) signals and between the CH_2 (30) and CO signal.

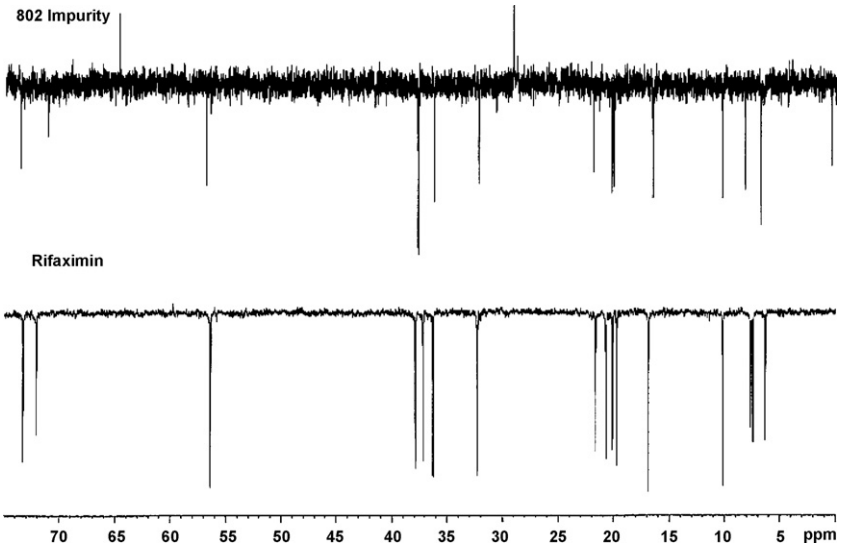
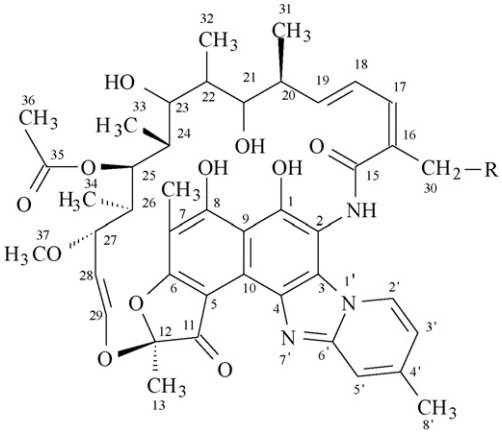


Fig. 6. APT ^{13}C NMR of 802 impurity and Rifaximin.

Table 1
^{1}H NMR chemical shifts of 802 impurity and Rifaximin



802 impurity R=OH		Rifaximin R=H [8]	
Proton(s)	δ (ppm)	Proton(s)	δ (ppm)
CH_3 (13)	2.02	CH_3 (13)	2.00
CH_3 (14)	2.16	CH_3 (14)	2.07
CH (17)	6.52	CH (17)	6.39
CH (18)	6.95	CH (18)	6.83
CH (19)	6.18	CH (19)	6.14
CH (20)	2.34	CH (20)	2.34
CH (21)	3.76	CH (21)	3.67
CH (22)	1.65	CH (22)	1.63
CH (23)	2.93	CH (23)	2.91
CH (24)	1.49	CH (24)	1.37
CH (25)	4.98	CH (25)	4.93
CH (26)	1.28	CH (26)	1.24
CH (27)	3.40	CH (27)	3.36
CH (28)	5.03	CH (28)	5.08
CH (29)	6.07	CH (29)	6.09
CH_2 (30)	**4.67–4.73**	**CH_3 (30)**	**2.23**
CH_3 (31)	0.78	CH_3 (31)	0.79
CH_3 (32)	0.99	CH_3 (32)	0.99
CH_3 (33)	0.38	CH_3 (33)	0.24
CH_3 (34)	−0.45	CH_3 (34)	−0.47
CH_3 (36)	1.94	CH_3 (36)	1.94
CH_3 (37)	3.04	CH_3 (37)	3.03
CH (2′)	8.77	CH (2′)	8.45
CH (3′)	7.15	CH (3′)	7.08
CH (5′)	7.45	CH (5′)	7.40
CH_3 (8′)	2.65	CH_3 (8′)	2.65

In bold the shits difference between C (30) hydrogens in Rifaximin and in Impurity 802 are evidenced.

4. Conclusion

The structural elucidation by UV, FT-IR, MS and NMR spectroscopy of an impurity (impurity 802), isolated from Rifaximin led to the conclusion that in this impurity a hydroxyl group is bonded to C_{30} position (Fig. 1). This structure is in close relation but does not fully agree with the structure reported in European Pharmacopoeia for Impurity H in which the hydroxyl group (probably erroneously) appears at C_{31} position. As the impurity 802 is structurally closely related to Rifaximin, the possibility of epimers and diastereoisomers was not investigated assuming that the formation of the impurity has not affected any of the stereogenic centers.

Acknowledgments

We are grateful to Interquim for the generous gift of Rifaximin industrial batches and to Dr. V. Bertacche for the impurity mass study.

References

[1] L. Cellai, M. Colosimo, E. Marchi, A.P. Venturini, G. Zanolo, Rifaximin (L/105), a new intestinal antibiotic: pharmacokinetic study after single oral administration of 3H-Rifaximin to rats, Chemioterapia 3 (1984) 373–377.
[2] A. Marchese, A. Salerno, A. Pesce, E.A. Debbia, G.C. Schito, In vitro activity of Rifaximin, metronidazole and vancomycin against Clostridium difficile and the rate of selection of spontaneously resistant mutants against representative anaerobic bacteria, including ammonia-producing species, Chemotherapy 46 (2000) 253–266.
[3] H.L. DuPont, Z.D. Jiang, P.C. Okhuysen, C.D. Ericsson, F.J. de la Cabada, S. Ke, M.W. DuPont, F. Martinez-Sandoval, A randomized, double-blind, placebo-controlled trial of Rifaximin to prevent travelers' diarrhea, Ann. Intern. Med. 142 (2005) 805–812.
[4] D.N. Taylor, R. McKenzie, A. Durbin, C. Carpenter, C.B. Atzinger, R. Haake, A.L. Bourgeois, Rifaximin, a nonabsorbed oral antibiotic prevents shigellosis after experimental challenge, Clin. Infect. Dis. 42 (2006) 1283–1288.
[5] V. Cannata, G.F. Tamagnone, Process for the synthesis of pyrido-imidazo rifamycins, PCT 4,557,866 Pubbl. (1985).
[6] Y. Huang, W. Qi, Z. Lu, Z. Liu, M. Goodfellow, Amycolatopsis rubida sp. Nov., a new Amycolatopsis species from soil, Int. J. Syst. Evol. Microbiol. 51 (2001) 1093–1097.
[7] European Pharmacopea 6th edition (6.5), 2009, p. 4955.
[8] M.R. Silverstein, G.C. Bassler, T.C. Morrill, Spectrometric Identification of Organic Compounds, 4th ed., John Wiley & Sons, 1981.
[9] C. Rossi, N. Marchettini, C. Bonechi, A. Donati, G. Corbini, P. Corti, NMR studies of a new class of Rifaximin-derived molecules: Rifaximin OR (Open Ring), J. Chem. Res. (S) (1996) 268–269.