



ELSEVIER

Journal of Chromatography A, 929 (2001) 51–61

JOURNAL OF
CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Radiosterilization of cefotaxime: investigation of potential degradation compounds by liquid chromatography–electrospray mass spectrometry

N. Barbarin^{a,*}, B. Tilquin^a, E. de Hoffmann^b

^aUniversité catholique de Louvain, Unité d'Analyse Chimique et Physico-chimique des Médicaments, UCL 72.30. Av. E. Mounier, 72, B-1200 Bruxelles, Belgium

^bUniversité catholique de Louvain, Unité de spectrométrie de masse, Pl. Pasteur, 1, B-1348 Louvain-la-Neuve, Belgium

Received 17 April 2000; received in revised form 15 May 2001; accepted 6 August 2001

Abstract

The nonvolatile radiolytic compounds produced by irradiation of cefotaxime were studied by a liquid chromatography–electrospray mass spectrometry method. Full scan LC–MS was first performed in order to obtain the m/z value of the protonated molecules of all detected peaks. LC–MS–MS was then carried out on the compounds of interest. A comparison between the MS–MS spectrum of cefotaxime and those of the radiolytic compounds showed that their fragmentation patterns were very similar suggesting that they were structural analogues of the main drug. The examination of the two main fragmentation pathways also permitted the location of the modified substructures. Moreover, it was shown that some stereoisomers appeared with the irradiation process. The complete fragmentation pattern of cefotaxime was studied by MSⁿ and used to obtain information about the structure of the radiolytic compounds. A complete structure was proposed for four of these. © 2001 Elsevier Science B.V. All rights reserved

Keywords: Radiosterilization; Cefotaxime

1. Introduction

Cefotaxime is an antibiotic that belongs to the cephalosporin group. It is still widely used because of its high activity against a large number of both gram-positive and gram-negative micro-organisms and its resistance to β -lactamases. All cephalosporin structures are based on the 7-aminocephalosporanic acid nucleus (called cephem nucleus) and vary by two side chains located in positions 3 and 7 of the central ring. The structure of cefotaxime is presented

in Fig. 1. This compound is commercialized as a sterile powder which requires dissolution prior to IV or IM administration.

The use of gamma irradiation to sterilize pharmaceuticals has recently become more and more popular. The main advantages of this process arise from the high penetrating power of the γ -rays allowing a terminal sterilization and from its isothermal character [1,2]. It is thus perfectly suitable for heat sensitive drugs like cephalosporins. On the other hand, irradiation treatment can produce new potentially toxic radiolytic products in very small amounts. Although the identification of these new compounds is not required in this case (because they

*Corresponding author. Fax: +32-2-764-7295.

E-mail address: tilquin@cham.ucl.ac.be (N. Barbarin).

2. Experimental

2.1. Materials

Cefotaxime sodium salt (Claforan[®]) was kindly provided by Hoechst. Methanol super-gradient grade was supplied by Lab-Scan (Dublin, Ireland) and ammonium acetate by Merck (Darmstadt, Germany). Water was deionized in the laboratory by a Milli-Q system (Millipore-Waters, Milford, MA, USA).

2.2. Irradiation

The irradiation was performed under an atmosphere of air and at ambient temperature in the panoramic ⁶⁰Co chamber in Louvain-la-Neuve (Catholic University of Louvain, Belgium). Cefotaxime was irradiated in solid state, at a dose of 25 kGy (1Gy corresponding to the absorption of 1 J/kg). The absorbed dose was measured using a Fricke dosimeter. The dose rate was 0.17 Gy/s.

2.3. LC–MS instruments and chromatographic conditions

The liquid chromatograph consisted of a TSP Spectra System P1000XR equipped with a Rheodyne Model 7725 manual injection and a 20- μ l loop. The mass detector was an LCQ[®] ion trap instrument with an electrospray ion source (Finnigan MAT, San Jose, CA, USA) controlled by the Xcalibur 1.0 software. The gas pressure was set as following: sheath gas: 65 p.s.i.; auxiliary gas: 15 p.s.i.

The chromatographic separation was performed on a 250 \times 2 mm Lichrospher[®] RP Select B column, 5- μ m particle size (Merck, Darmstadt, Germany). The mobile phase consisted of methanol and a 1 mM ammonium acetate aqueous solution. The pH was adjusted to 4 with HCl 0.1 M. The following gradient conditions were used: time=0': buffer/methanol (95:5); time=5': buffer/methanol (95:5); time=60': buffer/methanol (70:30). The flow-rate was set at 0.2 ml/min and entirely directed to the electrospray interface. The injection volume was 10 μ l of a 10⁻²M irradiated or nonirradiated cefotaxime solution. Analyses were performed at room temperature.

The following mass spectrometric conditions were used: capillary temperature: 200°C; source voltage:

5.2 kV; full scan from 80 to 1000 μ ; positive ion mode.

For LC–MSⁿ experiments, collision energy adjustment is represented in the text as a percentage of the maximum amplitude and noted “CID=x%”.

The study of the fragmentation patterns of cefotaxime was performed by directly injecting cefotaxime solutions into the electrospray source at 3 μ l/min using the syringe pump of the instrument.

3. Results and discussion

3.1. LC–MS

Fig. 2 presents the comparison of the total ions chromatograms of a non-irradiated and an irradiated sample of cefotaxime.

Electrospray being a soft ionization technique, the detected ions corresponded to the protonated molecules [M+H]⁺. Cefotaxime, for instance, with a molecular mass of 455 was detected at m/z 456. Similarly, other protonated molecules were located, as reported on the chromatograms of Fig. 2.

Fig. 2a shows that several impurities present before irradiation were detected along with the main compound. Those corresponding to m/z 414 and 398 have been previously identified as deacetylcefotaxime and deacetoxycefotaxime respectively [13,23]. Their molecular structures are presented in Fig. 1. These impurities, although not annotated in Fig. 2b, were also detected after irradiation with some 1-min shift in retention time. In addition, several additional peaks appeared with the irradiation. Their m/z values are annotated in Fig. 2b and are listed in Table 1. The inserts in Fig. 2 present extracted ion chromatograms of ions detected in the 40–60 min region before or after irradiation.

These results show that:

- Most radiolytic compounds produced protonated molecules with m/z close to that of cefotaxime suggesting similar molecular structures.
- Numerous isomeric compounds appeared with the irradiation: three protonated molecules at m/z 412, two at m/z 472 and two at m/z 486 were detected.
- An isomer of cefotaxime (detected at m/z 456) appeared with the irradiation. This compound has

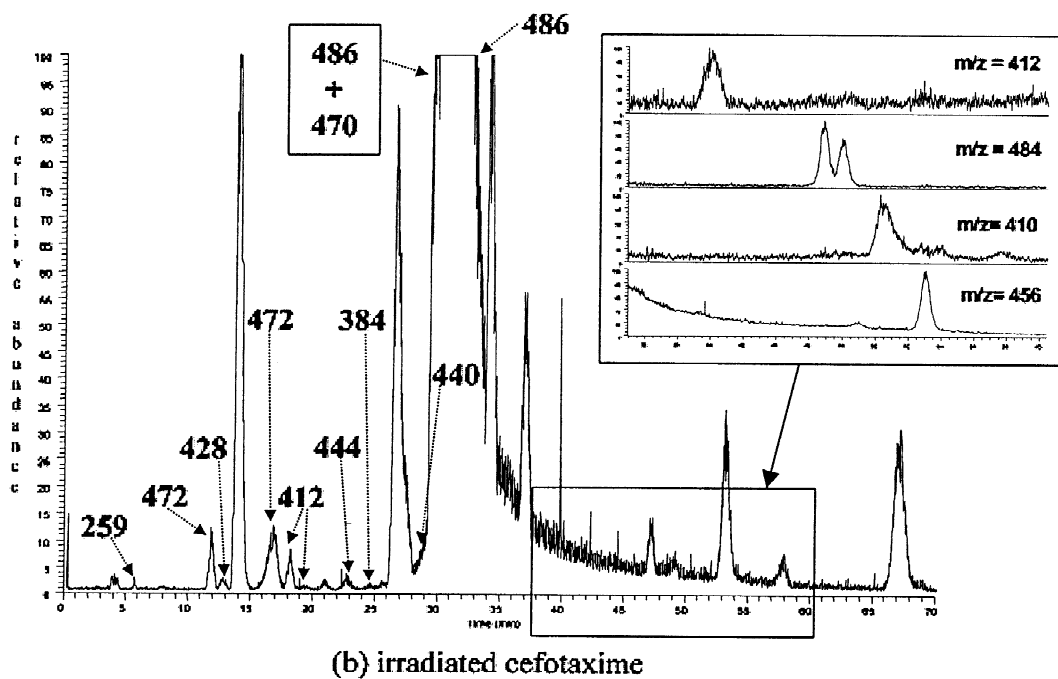
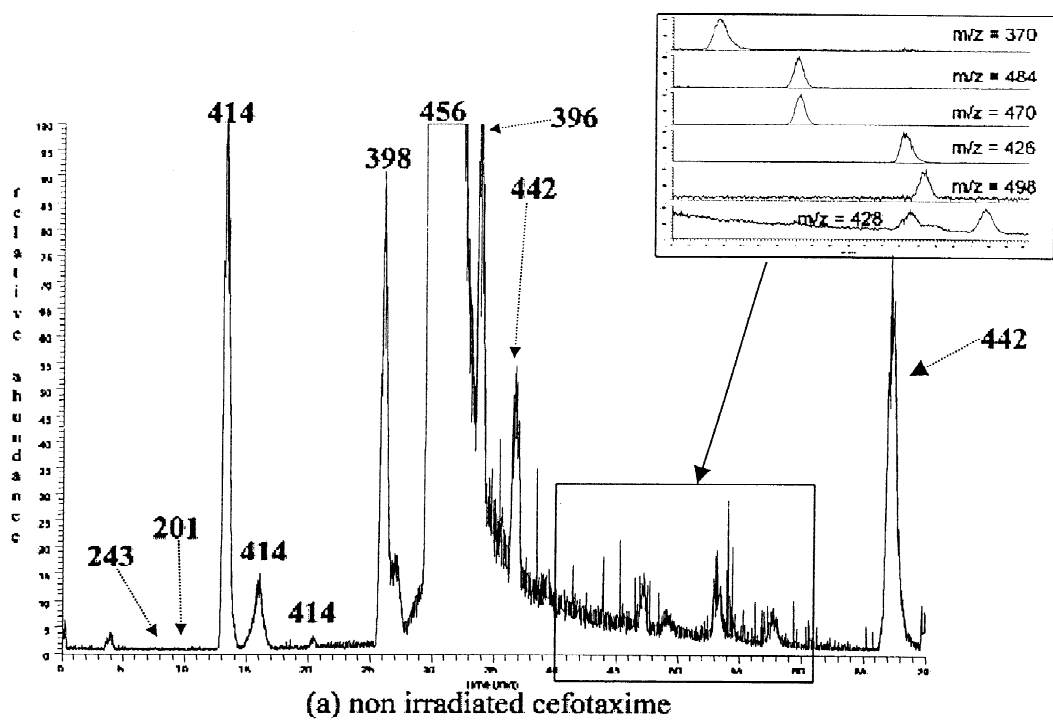


Fig. 2. Reconstructed ion chromatograms of (a) non irradiated and (b) irradiated cefotaxime with the corresponding m/z ratio of detected compounds.

Table 1
 m/z Value, main MS–MS fragments and hypothetical structure of the protonated degradation compounds produced by irradiation of cefotaxime

m/z Value of the protonated molecules	Main MS–MS fragments	Hypothetical structures
259	241, 213, 202, 185,175,158,144,126*, 116	?
472 (a)	454, 412, 394*, 366, 350, 322, 301, 290, 274, 225, 168	A _m -B _m -C
428	410, 379, 338, 335, 285, 241*, 227, 197, 182, 166, 156	A-B' _m
472 (b)	454*, 412, 366, 350, 322, 301, 290, 274, 225, 168	A _m -B _m -C
412 (a)	394, 285, 259, 241*, 227, 197, 182, 166, 126	A-B' _m
412 (b)	Not fragmented	?
444	426, 384*, 340, 306, 293, 224, 213, 126	A-B' _m
384	Not fragmented	?
440	380*, 366, 352, 336, 308, 261, 225, 167, 139	A _m -B-C
486 (a)	454, 426*, 398, 382, 354, 323, 307, 292, 271, 239, 167, 139	A _m -B-C
470	410*, 379, 366, 350, 338, 335, 307, 241, 197, 181, 166, 156	A-B _m -C
486 (b)	468, 426, 408*, 380, 333, 271, 253, 245, 196	A _m -B-C
412 (c)	394, 365, 285, 241*, 227, 210, 197, 182, 166, 126	A-B' _m
484	424*, 396, 378, 352, 334, 269, 241, 212, 184	A _m -B-C
410	350, 306*, 193, 156, 149, 126	A _m -B _m -C
456	396*, 368, 352, 324, 308, 277, 241, 216, 197, 139	Anticefo.

been previously identified by co-elution experiments as anticefotaxime [13,23]. It is the diastereoisomer “E” formed by cis–trans isomerization of the oxime function. Its molecular structure is presented in Fig. 1.

- An isomer (at m/z 484) of an impurity present before irradiation was detected in the irradiated sample (see inserts of Fig. 2).

3.2. LC–MS–MS

LC–MS–MS experiments were performed on cefotaxime, the two main impurities and on every $[M+H^+]$ molecules detected in Fig. 2b in order to verify the fragmentation pattern analogy between radiolytic compounds and cefotaxime. No effort was made to identify the unknown impurities present before irradiation.

3.2.1. LC–MS–MS on cefotaxime

The MS–MS spectrum of cefotaxime is presented in Fig. 3. Two main fragmentations, already described in the literature [19,24] were observed. The first one was an allylic cleavage with a loss of acetic acid (loss of a 60 μ) to give the ion at m/z 396. The second one was typical of all the β -lactam rings and corresponded to the cleavage of the cephem nucleus

with a loss of a neutral of 215 μ and the detection of an ion at m/z 241.

Consequently, cefotaxime could be divided into three substructures designated by letters from A to C as presented in the insert of Fig. 3. The substructure A corresponded to the ion at m/z 241, the substructure B designated the central part of the molecule and the substructure C corresponded to the acetyl group attached to the methyl located in position 3 of the cephem ring. Cefotaxime was thus represented as “A-B-C”.

3.2.2. LC–MS–MS on deacetylcefotaxime and deacetoxycefotaxime

The mass spectra of deacetylcefotaxime and deacetoxycefotaxime are presented in Fig. 4. The same allylic cleavage (with a loss of water) and the cleavage of the β -lactam ring were observed for deacetylcefotaxime (Fig. 4a). Therefore, this compound was represented as “A-B-C_m” with the subscript “m” indicating a modification in the substructure C.

The cleavage of the β -lactam ring was also observed for deacetoxycefotaxime which was represented as “A-B” as shown in Fig. 4b.

3.2.3. LC–MS–MS on the radiolytic compounds

The MS–MS spectra of the detected radiolytic

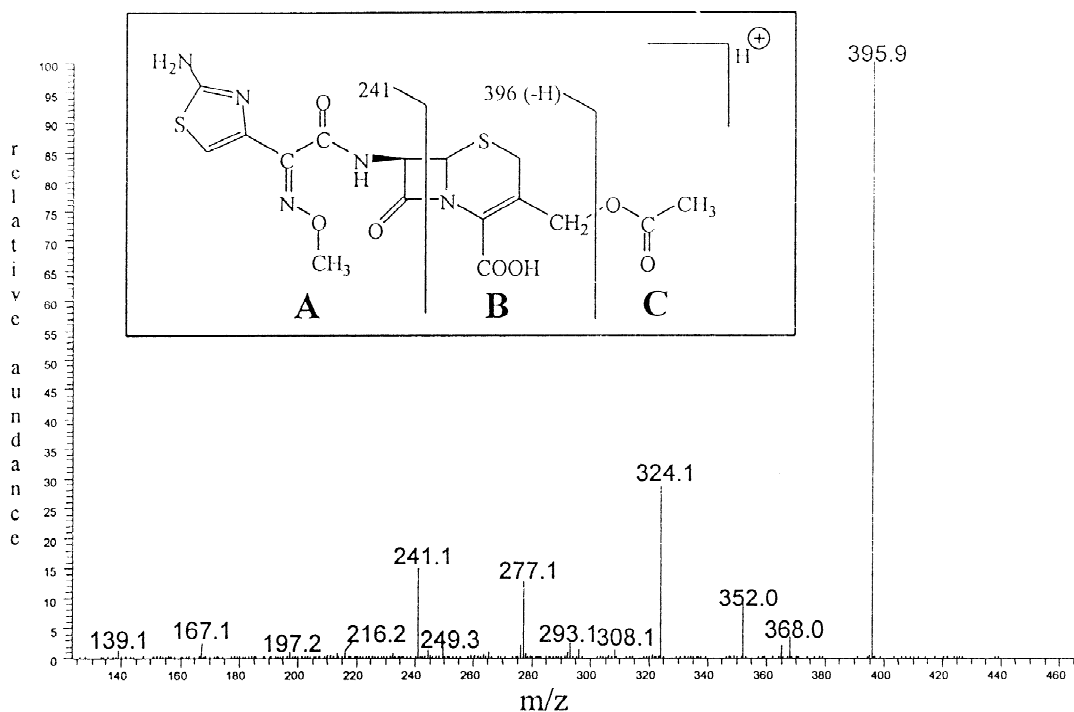


Fig. 3. MS–MS spectrum of the ion at m/z 456 corresponding to cefotaxime and the corresponding main fragmentation pattern. (CID: 20%).

compounds were similarly examined and their modified substructures were located. The results are summarized in Table 1.

The MS–MS spectra of the m/z 412 (a) and 412 (c) compounds were identical suggesting a diastereoisomeric relationship between them. On the contrary, the two m/z 486 compounds, having different MS–MS spectra, corresponded to different isobaric compounds. Finally, the two m/z 472 compounds showed identical fragments with different relative abundance and could be either diastereoisomers or isobaric compounds.

3.3. MS^n on cefotaxime

In order to understand better the fragmentation of the main compound, MS^n was performed by injecting a solution of cefotaxime directly into the LCQ mass spectrometer. In addition, MS^n was also carried out on the isotope of cefotaxime at m/z 458. The existence of this isotope was principally due to the presence of two sulphur atoms in the molecule which

could exist as ^{34}S and ^{32}S . The isotopical abundance of ^{34}S being 4.44%, the ion at m/z 458 represented approximately 8% of the main ion at m/z 456. A sensitivity limitation did not allow MS^n experiments on the radiolytic compounds.

MS^n on this “+2” isotope was used to know whether the fragmentation implied a loss of sulphur atoms. Three cases could be met: a fragmentation without any loss of sulphur atom: the daughter ions were detected with a m/z value of “+2” compared with those detected previously; a fragmentation with a loss of only one sulphur atom: two ions with a “normal” and a “+2” m/z value were detected with the same abundance since there was 50% chance to lose the ^{34}S ; a fragmentation with a loss of the two sulphur atoms: the daughter ions appeared with a m/z value identical to that detected previously.

The MS–MS spectrum of the ion at m/z 458 is presented in Fig. 5. As expected, the loss of acetic acid (loss of a neutral of 60 μ) led to a fragment at m/z 398 instead of the ion at m/z 396. The cleavage

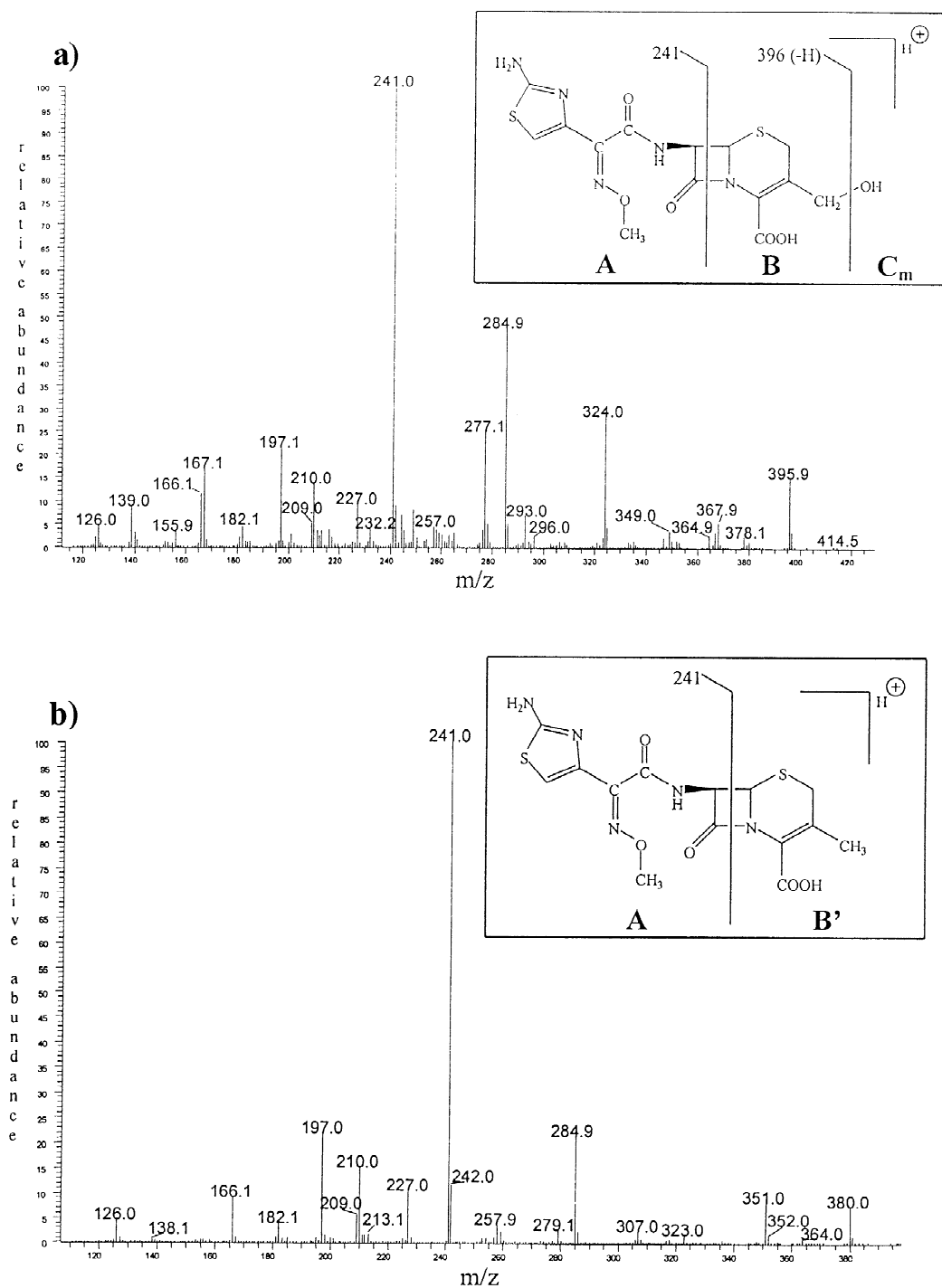


Fig. 4. MS-MS spectrum of (a) deacetylcefotaxime (at m/z 414) and (b) deacetoxycefotaxime (at m/z 398) and the corresponding main fragmentation patterns (CID: 20%).

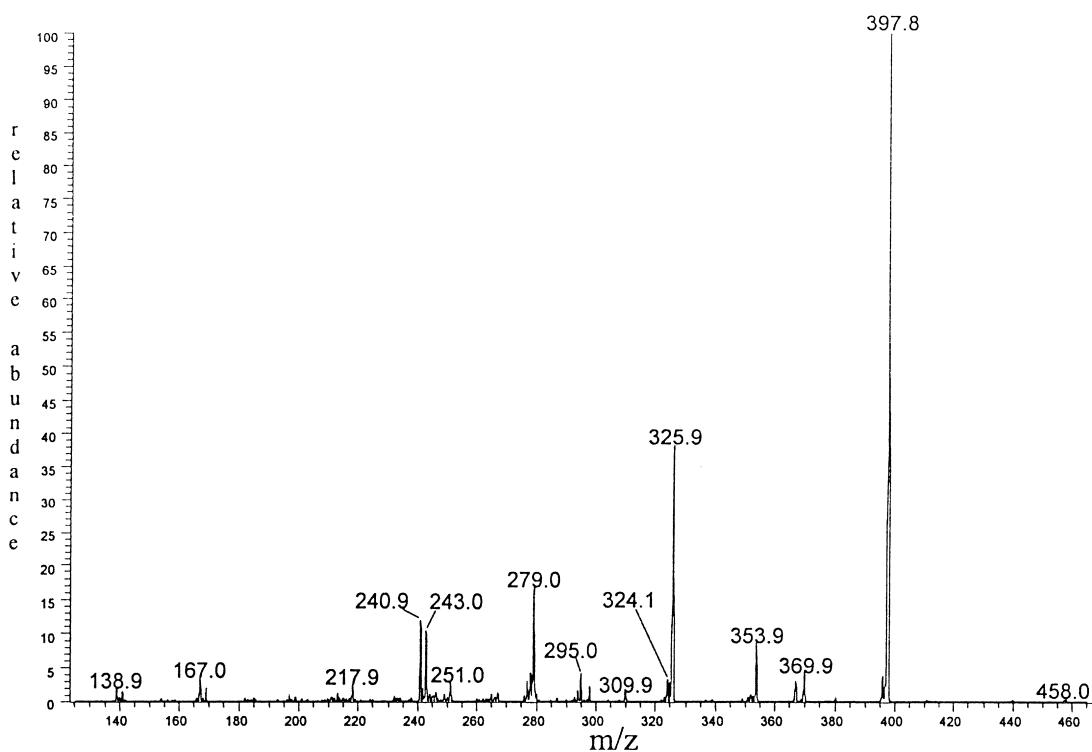


Fig. 5. MS–MS spectrum of the isotope “+2” of cefotaxime (at m/z 458). CID: 15%.

of the β -lactam ring led to the formation of two fragments at m/z 241 and 243 respectively, since it implied the loss of only one sulphur atom.

Data obtained by MSⁿ experiments on cefotaxime and its isotope ($m/z=458$) allowed the proposal of its fragmentation pattern as shown in Fig. 6 and the proposal of some fragmentation sequences presented in Fig. 7.

Fig. 7a presents the following fragmentation sequence: a loss of a neutral of 60 μ (acetic acid) with the formation of the ion at m/z 396; a loss of a neutral of 44 μ (CO_2) conducting to the fragment at m/z 352; a loss of 28 μ (CO) leading to the ion at m/z 324.

The loss of CO before CO_2 also happened from the ion at m/z 396 to give the ions at m/z 368 and 324 respectively. The subsequent fragmentation involved the consecutive loss of 157 and 28 μ leading to the ions at m/z 167 and 139 respectively, or the loss of 140 and 28 μ conducting to the fragments at

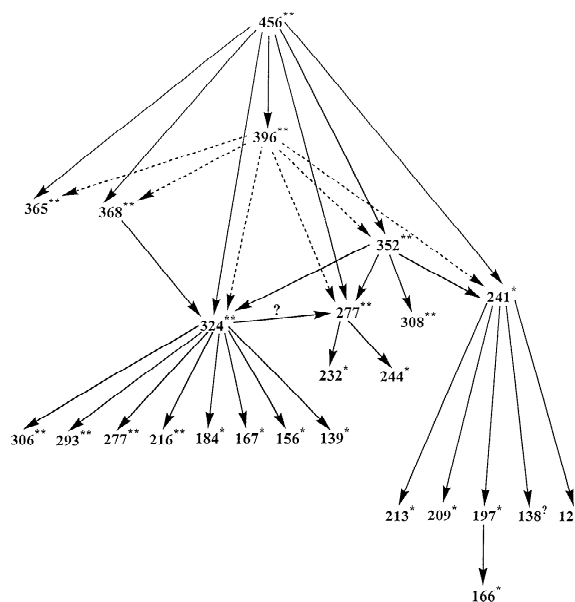


Fig. 6. Fragmentation pattern of cefotaxime. **=2 sulphur atoms; *=1 sulphur atom.

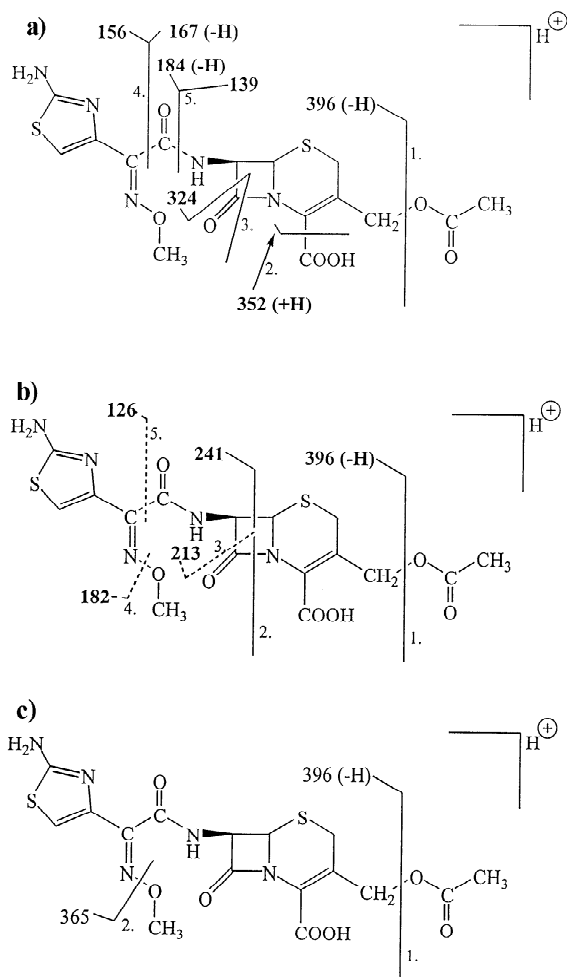


Fig. 7. Proposed fragmentation pathways of cefotaxime.

m/z 184 and 156. In these cases, the formation of fragments containing only one sulphur atom was confirmed by MS⁴ on the ion at m/z 326.

Another fragmentation pathway concerned the cleavage of the β -lactam ring from the ion at m/z 396 conducting to the fragment at m/z 241. The subsequent fragmentation involved consecutive loss of 28, 31, and 56 μ leading to the ions at m/z 213, 182 and 126 respectively as illustrated in Fig. 7b.

A last fragmentation pathway was proposed and is presented in Fig. 7c. It concerned the loss of 31 μ from the ion at m/z 396 leading to the fragment at m/z 365.

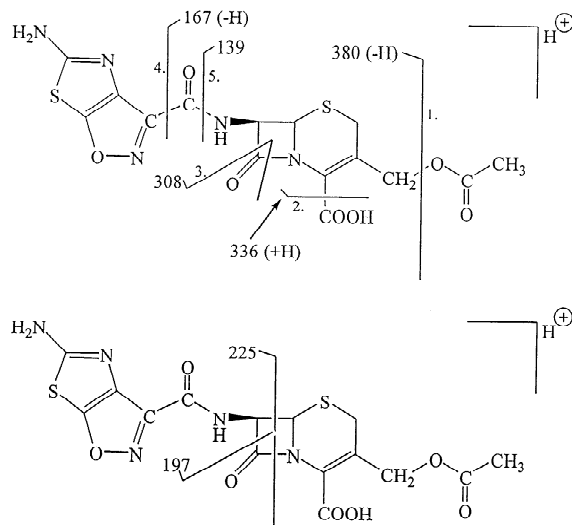


Fig. 8. Proposed structure and fragmentation patterns of the ion at m/z 440.

3.4. Characterization of radiolytic compounds

3.4.1. The ion at m/z 440

Fig. 8 proposes a structure for the component at m/z 440 along with its fragmentation pathway. Since no fragmentation concerning the modified part of the compound was detected, the structure remains hypothetical. However, the absence of loss of a 31- μ neutral from the ion at m/z 380, attributed, for cefotaxime, to a loss of the methoxy group of the oxime function was not detected in this case, suggesting a modification of this function. In addition, this function is known to be sensitive to gamma irradiation [13,25–27].

3.4.2. The ions at m/z 470 and 484

Fig. 9 presents hypothetical structures and the fragmentation patterns of the ions at m/z 470 and 484.

The structure proposed for the ion at m/z 470 is supported by the presence of a fragment at m/z 350 which could correspond to a loss of COS (60 μ) from the fragment at m/z 410. Indeed, COS was found to be a radiolytic volatile degradant of cefotaxime originating from the cephem ring [25,27].

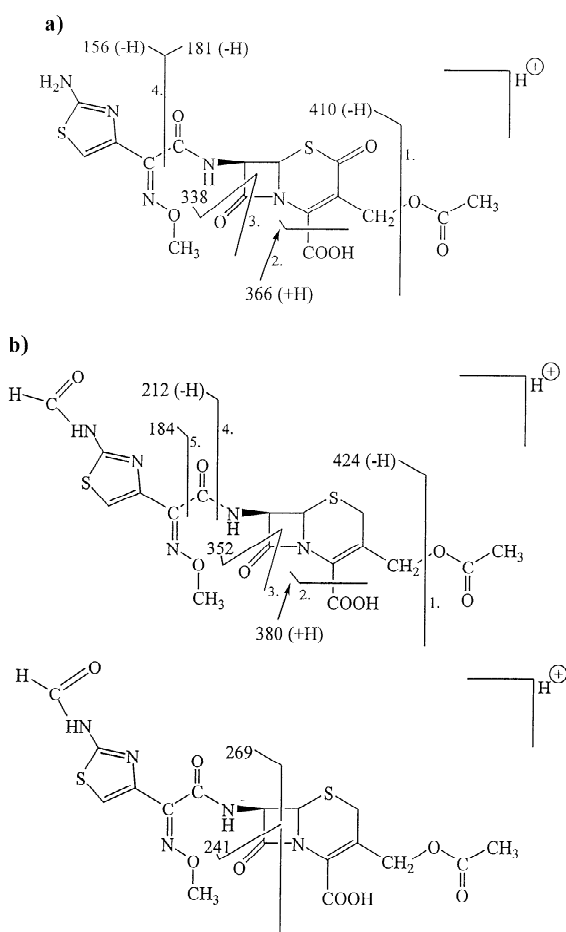


Fig. 9. Proposed structure and fragmentation patterns of the ions at (a) m/z 470 and (b) m/z 484.

The ion at m/z 484 had the same m/z value as an impurity detected before irradiation and could be its stereoisomer. Among the possible impurities of cefotaxime (listed in the European Pharmacopea 99 [28]), *N*-formylcefotaxime had a molecular mass and a structure that could perfectly justify the mass spectrum obtained for this compound. Its structure and fragmentation pattern are presented in Fig. 9b. Therefore, the radiolytic compound was proposed to be a stereoisomer of the *N*-formylcefotaxime. Unfortunately, mass spectrometry being in this case unhelpful to differentiate stereoisomers, the spatial structure of the compound remained unknown.

3.4.3. The other radiolytic compounds

The structures of the other radiolytic degradants were not elucidated. However, some information could be pointed out.

The compound with a m/z at 486 (a) was most likely modified at the part "A" (see Table 1) as suggested by the presence of the fragments at m/z 167 and 139, already detected for cefotaxime.

For the two compounds with a m/z of 444 and 410, the absence of a loss of 28 μ , attributed to the carbonyl group of the lactam function, suggested a modification of the cephem ring itself.

Finally, the three compounds with a m/z of 428, 412(a) and 412(b) presented mass spectra very similar to the one of deacetoxycefotaxime (with an abundant fragment at $m/z=241$ and the absence of the 60 μ loss). This suggested a modification of the side chain located in position 3 of the cephem ring.

4. Conclusions

The use of an on-line liquid chromatography–electrospray mass spectrometry method allowed the detection of the degradation compounds coming from the radiosterilization of cefotaxime even present in trace amounts (less than 0.1%). This direct method of detection permitted exclusion of any artifacts produced by isolation techniques.

These were found to be structural analogues of the main compound as suggested by the close m/z value of the protonated molecules $[M+H^+]$ and the similar main fragmentation patterns. Furthermore, the results suggested that some stereoisomers could be produced by irradiation.

The comparison between the MS–MS spectra of the radiolytic compounds and the fragmentation pathway of cefotaxime, established by multiple tandem mass spectrometry (MS^3) allowed the proposal of structures for three compounds besides anticefotaxime, already identified previously. The identity of these compounds should be confirmed by other techniques or by synthesis. Nevertheless, LC–MS can be very useful for the detection and characterization of radiolytic degradation compounds and the feasibility of radiosterilization can be further studied.

Acknowledgements

We gratefully thank Mr A. Spote and Mr R. Rozenberg for valuable assistance in performing LC–MS analyses.

References

- [1] G.P. Jacobs, *Isr. Pharm. J.* 25 (1985) 30.
- [2] B. Tilquin, *J. Pharm. Belg.* 46 (1991) 396.
- [3] ICH Harmonised Tripartite Guideline Q3A, Impurities in new drug substances, 1995
- [4] ICH Harmonised Tripartite Guideline Q3B, Impurities in new drug products, 1996
- [5] N. Barbarin, B. Tilquin, *Radiat. Phys. Chem.* 60 (2001) 359.
- [6] N.G.S. Gopal, *Radiat. Phys. Chem.* 12 (1978) 35.
- [7] G.P. Jacobs, P.A. Wills, *Radiat. Phys. Chem.* 31 (1988) 685.
- [8] C. Schüttler, K.W. Bögl, *J. Radiation Sterilisation* 1 (1992) 43.
- [9] C. Schüttler, K.W. Bögl, *J. Radiation Sterilisation* 1 (1993) 229.
- [10] C. Schüttler, K.W. Bögl, *J. Radiation Sterilisation* 1 (1994) 327.
- [11] C. Boëss, K.W. Bögl, *Drug Dev. Ind. Pharm.* 22 (1996) 495.
- [12] K. Tsuji, P.D. Rahn, K.A. Steindler, *J. Pharm. Sci.* 72 (1983) 23.
- [13] A.S. Crucq, B. Tilquin, *J. Pharm. Belg.* 51 (1996) 285.
- [14] F. Zeegers, M. Gibella, B. Tilquin, *Radiat. Phys. Chem.* 50 (1997) 149.
- [15] S. Suwanrumpha, R.B. Freas, *Biomed. Mass Spectrom.* 18 (1989) 983.
- [16] R.D. Voyksner, *J. Chromatogr.* 567 (1991) 389.
- [17] H.T. Pan, P. Kumari, J.A.F. De Silva, C.C. Lin, *J. Pharm. Sci.* 82 (1993) 52.
- [18] K. Kobayashi, K. Sato, Y. Mizuno, Y. Katsumata, *J. Chromatogr. B* 677 (1996) 275.
- [19] C.E. Parker, J.R. Perkins, K.B. Tomer, *J. Chromatogr.* 616 (1993) 45.
- [20] K.L. Tyczkowska, R.D. Voyksner, R.F. Straub, A.L. Aronson, *J. Assoc. Off. Anal. Chem.* 71 (1994) 1122.
- [21] R. Straub, M. Linder, R.D. Voyksner, *Anal. Chem.* 66 (1994) 3651.
- [22] L. Rovatti, M. Garzotti, A. Casazza, M. Hamdab, *J. Chromogr. A* 819 (1999) 133.
- [23] A.S. Crucq, *Mécanismes radicalaires des céphalosporines irradiées*. Thesis (1995), Université Catholique de Louvain (UCL), Bruxelles.
- [24] R.F. Straub, R.D. Voyksner, *J. Chromatogr.* 647 (1993) 167.
- [25] N. Barbarin, A.S. Crucq, B. Tilquin, *Radiat. Phys. Chem.* 48 (1996) 787.
- [26] A.S. Crucq, B. Tilquin, *Free Radic. Biol. Med.* 21 (1996) 827.
- [27] N. Barbarin, B. Rollmann, B. Tilquin, *Int. J. Pharm.* 178 (1999) 203.
- [28] *Eur. Pharm. Addendum*, (1999) 363.