Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 790–796
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Identification of degradation products of diclofenac by electrospray ion trap mass spectrometry

Marie-Josèphe Galmier[a,b,*], Bernadette Bouchon[a,b], Jean-Claude Madelmont[b], Fabrice Mercier[c], Frédéric Pilotaz[c], Claire Lartigue[a,b]

[a] *Laboratoire de Chimie Analytique et de Spectrométrie de Masse, UFR de Pharmacie, Place Henri Dunant, B.P. 38, F- 63001 Clermont-Ferrand Cedex, France*
[b] *UMR INSERM 484, rue Montalembert, B.P. 184, F-63001 Clermont-Ferrand Cedex, France*
[c] *Laboratoires Théa, 12 rue Louis Blériot, ZI du Brézet, F-63017 Clermont-Ferrand Cedex 2, France*

Received 5 November 2004; received in revised form 8 February 2005; accepted 14 February 2005
Available online 17 March 2005

Abstract

The degradation products of diclofenac in aqueous dosage form in accelerated storage conditions were characterized by electrospray ionization–ion trap mass spectrometry (ESI–MS). Liquid chromatography (LC)–MS analyses revealed the presence of three degradation products. ESI–MS^n spectra were used to study diclofenac fragmentation in detail and to characterize the structures of degradation products. A previously described degradation product, formed by a cyclization reaction of diclofenac producing the indolinone derivative, was found. As any hydroxylated product was found, no oxidation seems to occur in the dosage form used. On the contrary, two degradates have been detected and identified, leading to a primary alcohol structure or an aldehyde function in place of the acetate group of diclofenac.

Keywords: Diclofenac; Degradation products; Electrospray ionization–ion trap mass spectrometry; Liquid chromatography–mass spectrometry

1. Introduction

Diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid, sodium salt, is a synthetic non steroidal anti-inflammatory drug. This drug is well known and commercially available on the market in various pharmaceutical forms (enteric coated oral tablets, sustained releases oral tablets, topical preparations, ophthalmics, injections, ...). Stability studies of the dosage form have shown the presence of degradation products. Drug degradation in dosage forms is a very complex and often unpredictable process and its mechanism varies from compound to compound. Depending on the structure of the drug molecule, degradation process may be influenced by temperature, humidity, light, containers and occurs from oxidation processes (air, light and metal ions traces), hydrolysis, dehydration, adduct formation, dimerization, rearrangement, excipient reaction, and often from the combination of these processes. During the past decade, the LC–MS system has become one of the most powerful techniques for the identification of degradation products in dosage formulations [1–3]. For the determination of diclofenac and its metabolites or degradation products, various analytical methods have been used including LC/UV [4–8], LC/APCI–MS [9], LC/ICP–MS/TOF–MS [10], LC/ICP–MS [11], LC/ESI–MS in negative mode [12], HPTLC [13], GC/MS[14] and CE [5]. In the present work, LC/ESI–MS in a positive mode and ion-trap MS^n experiments were used to characterize the degradation products of diclofenac in aqueous solution.

2. Experimental

2.1. Reagents and chemicals

Diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid, sodium salt, was supplied by Thea Laboratories

* Corresponding author. Tel.: +33 473178067; fax: +33 473263874.
E-mail address: galmier@inserm484.u-clermont1.fr (M.-J. Galmier).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.02.016

(Clermont-Ferrand, France). 1-(2,6-Dichlorophenyl)indolin-2-one was purchased from European Pharmacopoeia (Strasbourg, France), [2-[(2,6-dichlorophenyl)amino]phenyl]methanol and 2-[(2,6-dichlorophenyl)amino] benzaldehyde were from LGC Promochem (Molsheim, France). HPLC grade methanol was purchased from SDS (Peypin, France) and formic acid from Sigma (Saint-Quentin Fallavier, France). Pharmaceutical ophthalmic solutions of diclofenac sodium at 1 mg mL^{-1} were manufactured by Thea laboratories. The main excipient of this formulation was a polyoxyethylated castor oil (Cremophor® EL–BASF AG., Ludwigshafen, Germany) and the final buffered aqueous solution was at neutral pH.

2.2. Analytical methods

A HP 1100 (Agilent, Palo Alto, CA, USA) liquid chromatograph equipped with a 500 μm × 150 mm Kromasil C18 capillary column, particle size 5 μm (AIT, Le Mesnil le Roi, France) was used in the separation of diclofenac and its degradation products. The mobile phase, a mixture of methanol and 0.1% aqueous formic acid pH 2.5 (80:20, v/v), was degassed and filtered through a 0.45 μm filter (Millipore, Saint-Quentin en Yvelines, France). LC separations were performed at room temperature at a flow rate of 0.4 mL min^{-1}. The eluent flow from the LC pump was split 1:100 with a LC-Packings (Dionex, France) pre-column splitter. Detection of compounds was performed using a UV detector at 254 nm and on-line mass spectrometry.

Mass spectrometric experiments were performed in a positive ion mode on an Esquire-LC ion trap (Bruker, Bremen, Germany) mass spectrometer equipped with an ESI source; the end plate voltage was set at −3.5 kV, the capillary at −4 kV, the capillary exit at 30 V and the skimmer at 10 V. The samples, diluted 1/30 in methanol/water (1:1, v/v) containing 0.1% formic acid, were introduced by injection with a Rheodyne injector with a 5 μL sample loop. Nitrogen was used as the drying (300 °C, 5 L min^{-1}) and nebulizing (17 psi) gas. The mass range scanned in MS and MS^n runs was *m/z* 50–500. Each MS spectrum was recorded by averaging 10 spectra. Detailed fragmentation studies of diclofenac sodium and specified degradation products were performed by ESI–MS direct infusion into the spectrometer at a flow of 2 μL min^{-1} using a microsyringe pump (Harvard Apparatus, USA). MS analyses were performed using total ion current (TIC) chromatograms. Specific compounds (*m/z* corresponding to diclofenac and/or specified degradation products) were detected using extracted ion current (EIC) chromatograms. Electron ionization (EI) spectra were obtained by direct introduction using a HP-5989 A mass engine (Hewlett Packard/Agilent, Palo Alto, CA, USA).

2.3. Samples preparation

Pharmaceutical ophthalmic solutions of diclofenac sodium at 1 mg mL^{-1} were stored in glass vials under accelerated testing conditions for 9 weeks at 60 °C prior to analysis. No degraded samples were stored at room temperature.

3. Results and discussion

Diclofenac degradation products were separated by high performance liquid chromatography (HPLC) and characterized by on-line electrospray ionization–ion trap mass spectrometry (ESI–MS). The comparison of chromatograms obtained with degraded samples and non degraded samples revealed the presence of three additional peaks in the degraded samples: DP1 at 17.5 min (*m/z* 278/280), DP2 at 21 min (*m/z* 268/270) and DP3 at 27 min (*m/z* 266/268). Fig. 1(a) shows EIC chromatograms and Fig. 1(b) the ESI–MS^2 mass spectra of diclofenac and degradates.

3.1. Diclofenac fragmentation pattern

The characteristic pattern of diclofenac fragmentation was used to characterize the degradation products of diclofenac and MS and MS^2 spectra are shown in Fig. 2. The presence of chlorine and its characteristic isotopic species (^{35}Cl and ^{37}Cl) in diclofenac allowed to follow the diclofenac degradation products (DP). Indeed, isotopic peaks at M, M + 2 and M + 4 with relative intensities in a ratio 9:6:1 are characteristics of the presence of two chlorine atoms as observed in the protonated diclofenac mass spectrum (ions 296/298/300). In the following discussion, only the most abundant isotopes are considered, which leads to a proportion of 3:2 for M and M + 2. The compound ion spectrum at *m/z* 278/280 from protonated diclofenac $[M+H]^+$ is due to the loss of 18 u and corresponds to dehydration (Scheme 1). The fragmentation of ions *m/z* 278/280 (ratio 3:2) $[M+H-H_2O]^+$ shows the loss of a neutral CO (28 u) producing cations at *m/z* 250/252 (3:2) $[M+H-H_2O-CO]^+$. Further cleavage of chlorine radical leads to the radical ions *m/z* 215/217 (3:1) $[M+H-H_2O-CO-{}^{\bullet}Cl]^{\bullet+}$. The MS^3 fragmentation of *m/z* 215/217 shows the loss of a second chlorine radical producing the cation at *m/z* 180 $[MH-H_2O-CO-2{}^{\bullet}Cl]^+$.

Interestingly, the formation of a radical ion from a cation is observed in the ion trap MS^n fragmentation, on the contrary with classical mechanisms of electron impact fragmentation in which this secondary fragmentation (a cation leading to a radical-cation) is considered as highly improbable. This was confirmed by acquiring the mass spectra of diclofenac in EI mode and the series of ions was 295/297 $[M]^{\bullet+} \rightarrow$ 277/279 $[M-H_2O]^{\bullet+} \rightarrow$ 242/244 $[M-H_2O-{}^{\bullet}Cl]^+ \rightarrow$ 214/216 $[M-H_2O-{}^{\bullet}Cl-CO]^+$. The ion at *m/z* 214 is the base peak in the EI spectrum and its high stability can be explained by the extensive charge delocalization and stabilization as a result from the formation of a quaternary ammonium ion as observed for *m/z* 215/217 in ESI–MS (Scheme 1).

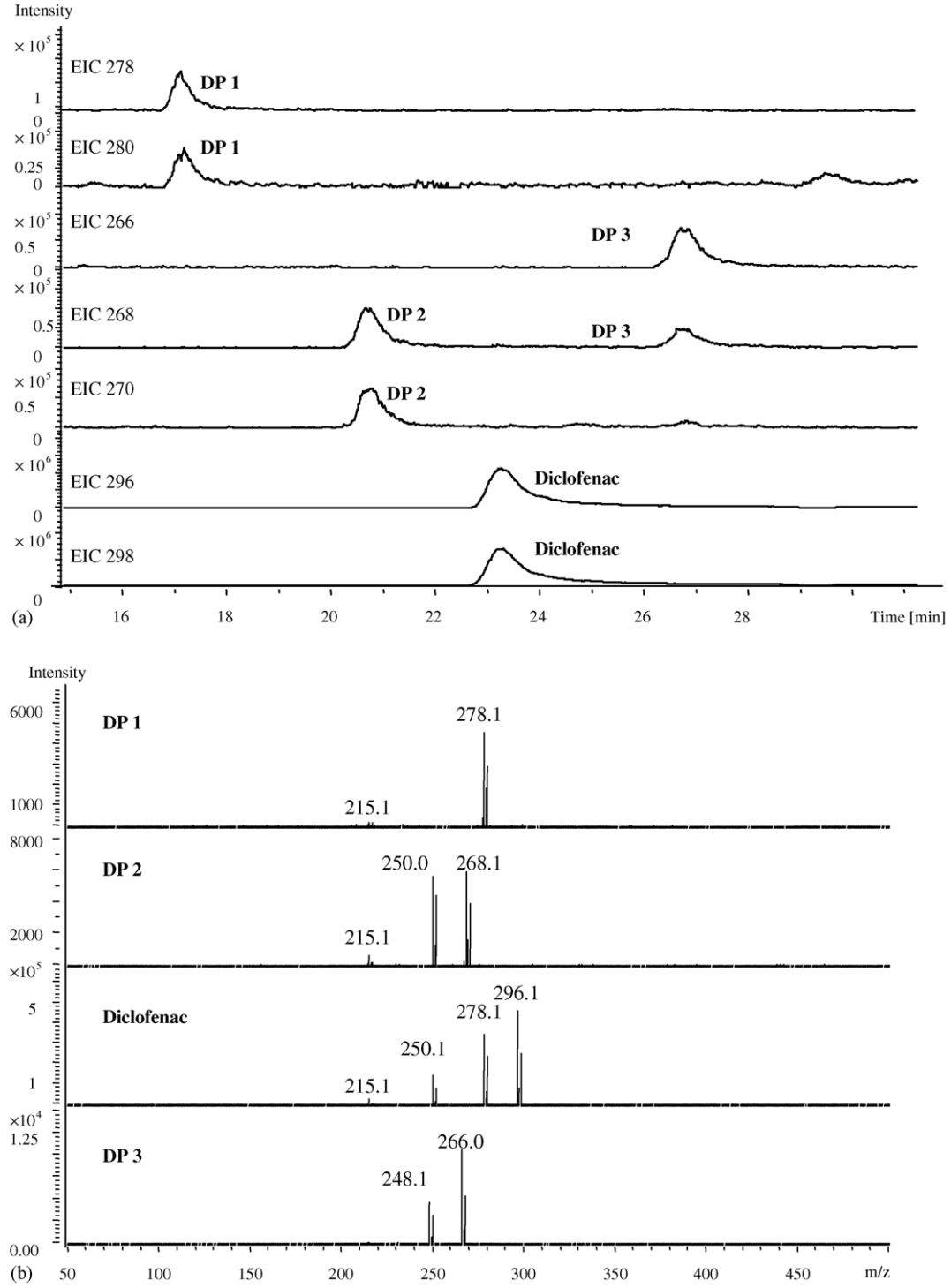


Fig. 1. LC–MS analysis of a diclofenac pharmaceutical aqueous formulation stored for 9 weeks at 60 °C: (a) extracted ion current (EIC) chromatograms of diclofenac and its degraded products at [M] and [M + 2] for each compound, with regard to the isotopics species of chlorine (^{35}Cl and ^{37}Cl): *m/z* 278 and 280 for DP1 at 17.5 min, *m/z* 268/270 for DP2 at 21 min, *m/z* 296/298 for diclofenac at 23.2 min and *m/z* 266/268 for DP3 at 27.0 min. (b) LC–ESI–MS^2 spectra of the chlorinated ions.

3.2. Diclofenac degradation product 1

After isolation and fragmentation of the precursor ion at *m/z* 278/280 (ratio 3:2) of the DP1 peak, fragment ions at 243/245 (3:1) and 215/217 (3:1) are obtained. The difference of 18 u between DP1 and diclofenac is consistent with a dehydration of diclofenac by an intramolecular cyclization leading to the 1-(2,6-dichlorophenyl)indolin-2-one. This compound is generally described as a synthesis intermediate of diclofenac (impurity A of the European pharmacopoeia)

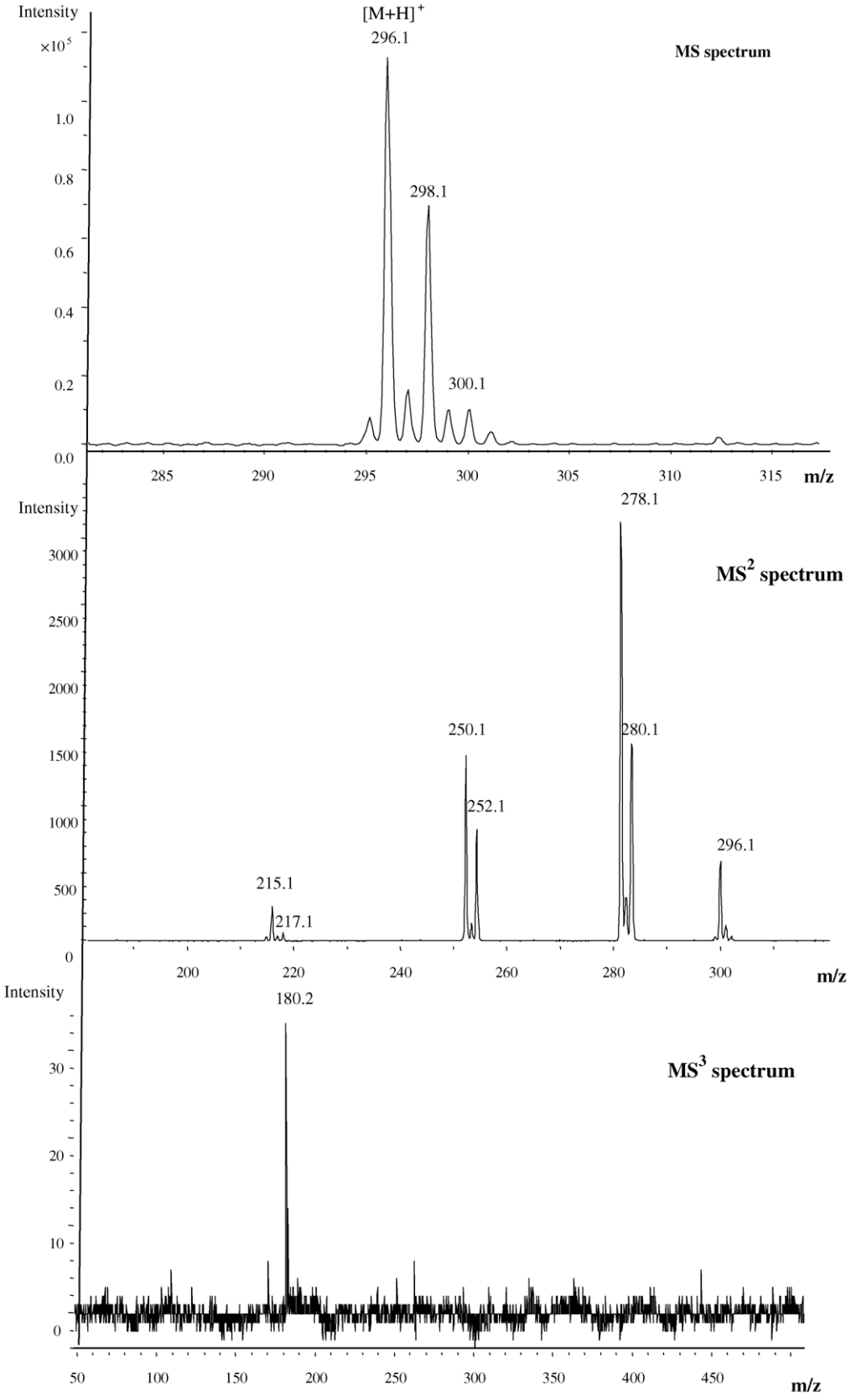
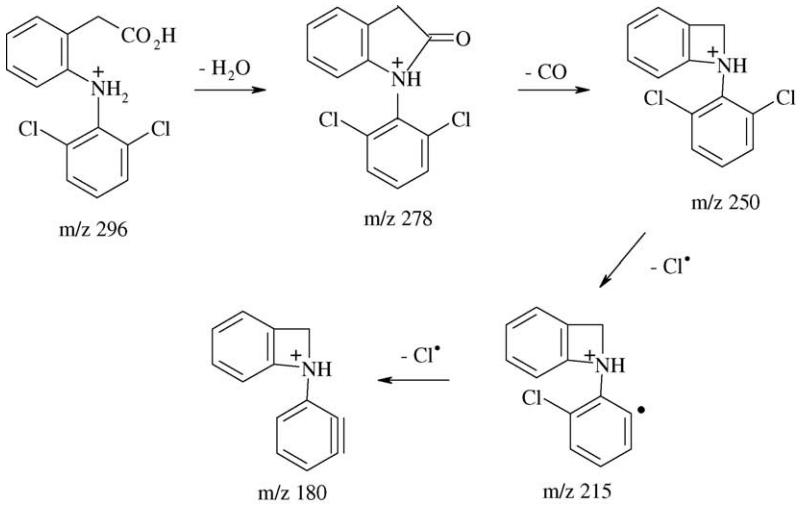


Fig. 2. ESI mass spectra of diclofenac reference standard. MS spectrum (top), MS^2 spectrum of *m*/*z* 296/298 (middle) and MS^3 spectrum of *m*/*z* 215/217 (bottom).



Scheme 1. Proposed fragmentation mechanism for protoned diclofenac.

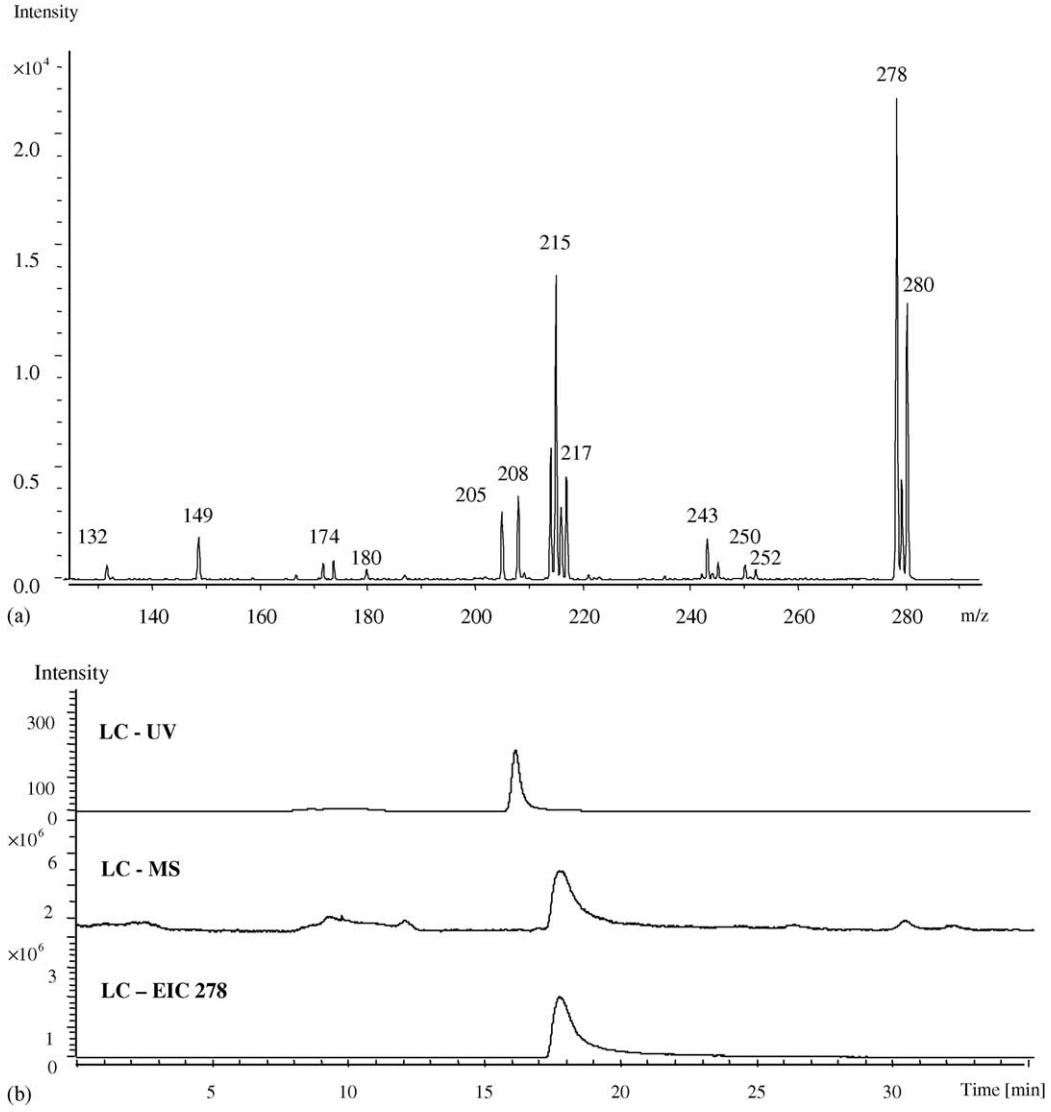
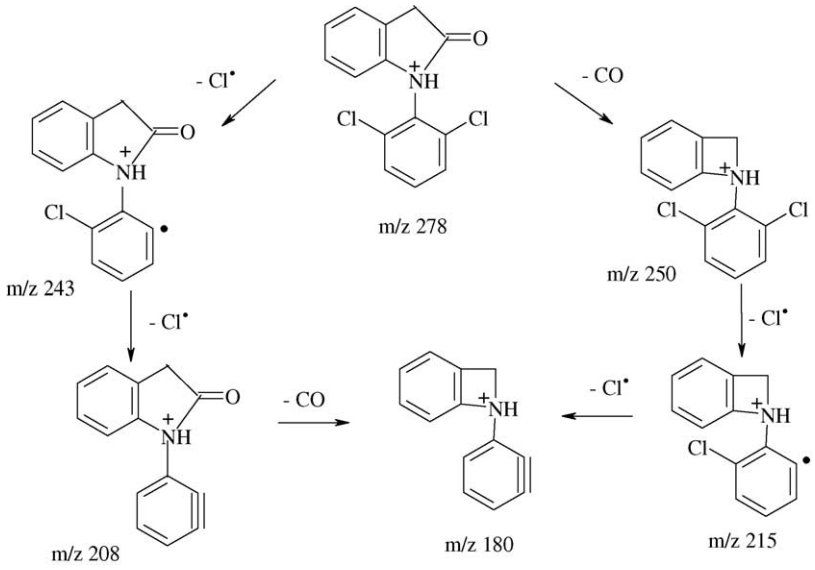


Fig. 3. Analysis of [1-(2,6-dichlorophenyl)indolin-2-one] standard (a) ESI–MS^2 spectrum obtained by fragmentation of the $[M+H]^+$ ion by direct infusion. (b) LC–MS analysis of the same solution. UV was measured at 254 nm. The shift between UV and MS elution time is due to the on-line analysis.



Scheme 2. Proposed fragmentation mechanism for protoned DP1.

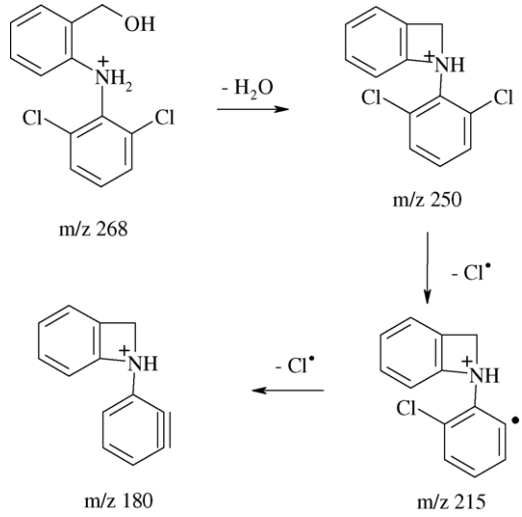
[15] but also appears after heating [16–19] or light exposure [8] and was already described as a degradation product in topical emulgel [7]. The fragmentation pattern of the corresponding standard was determined by direct analysis and Fig. 3(a) shows the following series of MS^2 product ions: 278/280, 250/252, 243/245, 215/217 and 208. A MS^3 experiment displayed in fact two different pathways (Scheme 2). The first one is characterized by an initial CO loss from *m/z* 278/280 $[M+H]^+$ leading to *m/z* 250/252 (ratio 3:2) $[M+H-CO]^+$ and the second pathway is an initial loss of a chlorine radical leading to *m/z* 243/245 (ratio 3:1) $[M+H-{}^{\bullet}Cl]^{\bullet+}$. The fragmentation of *m/z* 243/245 shows the loss of a second chlorine radical leading to *m/z* 208 followed by the loss of a neutral CO leading to *m/z* 180. Fig. 3(b) shows the measured UV chromatogram, the ESI–MS–TIC and the ESI–MS–EIC *m/z* 278. As retention time of reference is similar to those of the DP1, this degraded product of diclofenac in the dosage form is therefore confirmed as being 1-(2,6-dichlorophenyl)indolin-2-one.

3.3. Diclofenac degradation product 2

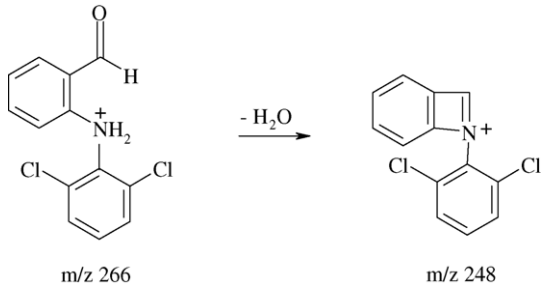
The mass spectrum of protonated DP2 shows ions at *m/z* 268/270 in a ratio 3:2 characteristic of two chlorine atoms. The difference of 28 mass units between diclofenac and DP2 suggests a primary alcohol structure in DP2 instead of the acetate group of diclofenac. The product ions spectrum of protonated DP2 in Fig. 1(b), produced by an MS^2 experiment, shows the ions *m/z* 250/252 (3:2) due to a dehydration and the subsequent loss of a chlorine radical producing ions *m/z* 215/217 (3:1). The MS^3 fragmentation of *m/z* 215/217 shows the loss of a second chlorine radical leading to *m/z* 180 (Scheme 3). These results are consistent with the structure of the impurity C described in the European Pharmacopoeia [15]. Further HPLC experiment with the reference product of impurity C confirmed the identity of DP2 as [2-[(2,6-dichlorophenyl)amino]phenyl]methanol.

3.4. Diclofenac degradation product 3

The mass spectrum of protonated DP3 shows ions at *m/z* 266/268 in a ratio 3:2 (two chlorine atoms). The difference of 30 mass units between diclofenac and DP3 suggests an aldehyde structure in DP3 instead of the acetate group of diclofenac. The MS^2 fragmentation of protonated DP3 leads to *m/z* 248/250 by loss of 18 u by dehydration in Fig. 1(b). The structure of DP3 seems to be in keeping with the impurity B structure described for diclofenac in European Pharmacopoeia [15]. After direct infusion of this standard, the MS^2 analysis leads to *m/z* 248/250 as for DP3. The dehydration leads to a quaternary ammonium structure with a higher



Scheme 3. Proposed fragmentation mechanism for protoned DP 2.



Scheme 4. Proposed fragmentation mechanism for protoned DP 3.

degree of conjugation than in *m/z* 250/252 observed in diclofenac, DP1 and DP2. Moreover, any subsequent fragmentation was observed by using MS^3 analysis for DP3 as for impurity B (Scheme 4). Further HPLC showed the concordance in the retention times and confirmed the identity of DP3 as 2-[(2,6-dichlorophenyl)amino]benzaldehyde.

4. Conclusion

Ion trap with MS^n spectra analysis has been shown to be a very powerful tool in the characterization of the degradation products of diclofenac. The method is based on comparison of the fragmentation pathways of parent molecule and degradation products. The degradate formed by an intramolecular cyclization with loss of water has been previously described [19]. Two other degradation products have been found in accelerated storage conditions (9 weeks at 60 °C). In the contrary with attempted results with regard to hydroxylated compounds described after UV/H_2O_2 and ozone exposure [20], no advanced oxidation was found. DP2 and DP3 degradates, identified as the impurities B and C of diclofenac in the European Pharmacopeia monograph, seem to proceed in a reductive way which might be related with the Chremophor® EL based vehicle, a surfactant agent used to improve the solubility of the drug substance. At last, these products B and C, known to be synthesis impurities, have never been described as degradation products in a dosage form.

References

[1] K.J. Volk, S.E. Klohr, R.A. Rourick, E.H. Kerns, M.S. Lee, J. Pharm. Biomed. 14 (1996) 1663–1674.
[2] Y. Wu, Biomed. Chromatogr. 14 (2000) 384–396.
[3] K. Pennanen, T. Kotiaho, K. Huikko, R. Kostiainen, J. Mass Spectrom. 36 (2001) 791–797.
[4] H. Ye, M. Zhang, Chinese Pharm. J. 35 (3) (2000) 192–194.
[5] M.S. Aurora-Prado, M. Steppe, M.F.M. Tavares, E.R.M. Kedor-Hackmann, M.I.R.M. Santoro, J. AOAC Int. 85 (2) (2002) 330–340.
[6] L. Seng-Chung, T. Tung-Hu, J. Chromatogr. B 769 (2) (2002) 351–356.
[7] R. Hajkova, P. Solich, M. Pospigilovad, J. Sicha, Anal. Chim. Acta 467 (1/2) (2002) 91–96.
[8] M.C. Gaudiano, L. Valvo, P. Bertocchi, L. Manna, J. Pharm. Biomed. 32 (2003) 151–158.
[9] M.E. Abdel-Hamid, L. Novotny, H. Hamza, J. Pharm. Biomed. 24 (4) (2001) 587–594.
[10] O. Corcoran, J.K. Nicholson, E.M. Lenz, F. Abou-Shakra, J. Castro-Perez, A.B. Sage, I.D. Wilson, Rapid Commun. Mass Spectrom. 14 (2000) 2377–2384.
[11] C.J. Duckett, N.J.C. Bailey, H. Walker, F. Abou-Shakra, I.D. Wilson, J.C. Lindon, J.K. Nicholson, Rapid Commun. Mass Spectrom. 16 (2002) 245–247.
[12] X.S. Miao, B.G. Koenig, C.D. Metcalfe, J. Chromatogr. A. 952 (2002) 139–147.
[13] L.G. Lala, P.M. D'Mello, S.R. Naik, J. Pharm. Biomed. 29 (2002) 539–544.
[14] R. Andreozzi, M. Raffaele, P. Nicklas, Chemosphere 50 (2003) 1319–1330.
[15] European pharmacopoeia, 5th ed., Direction de la Qualité du Médicament du Conseil de l'Europe (2) (2005) 1532–1534.
[16] M. Zajac, B. Stanisz, W. Musial, Acta Pol. Pharm. Drug Res. 55 (5) (1998) 371–374.
[17] M.E. Palomo, M.P. Ballesteros, P. Frutos, J. Pharm. Biomed. 21 (1) (1999) 83–94.
[18] P. Tudja, M.Z.I. Khan, E. Mestrovic, M. Horvat, P. Golja, Chem. Pharm. Bull. 49 (10) (2001) 1245–1250.
[19] J. Roy, M. Islam, A.H. Khan, S.C. Das, M. Akhteruzzamam, A.K. Deb, A.H. Mahbub Alam, J. Pharm. Sci. 90 (5) (2001) 541–544.
[20] D. Vogna, R. Marotta, A. Napolitano, R. Andreozzi, M. Ischia, Water Res. 38 (2) (2004) 414–422.