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Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 651–657

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Isolation and identification of a degradation product in a capsule formulation containing the elastase inhibitor, DMP 777

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Received 23 May 2000; received in revised form 12 September 2000; accepted 12 September 2000

Abstract

An unexpected degradation product, greater than 0.10%, was observed in a DMP 777 capsule formulation stored at 40°C/75% r.h. for 3 months and 25°C/60% r.h. for 2 years. The degradant of interest was prepared in quantity by refluxing the drug substance in dilute acid. A preparative HPLC method was developed to separate the various degradants and to collect each as a separate fraction. Each fraction was analyzed by the analytical HPLC gradient test method to assure positive identification of each peak and to correlate each peak to the original capsule sample. Key isolated degradation products were used for structure elucidation with mass spectrometry and NMR. The major degradant of interest in the capsule formulation was found to be a carboxylic acid resulting from the acid hydrolysis of an amide bond. © 2001 Published by Elsevier Science B.V. All rights reserved.

Keywords: Preparative chromatography; Elastase inhibitor; Acid hydrolysis; Mass spectrometry; Nuclear magnetic resonance; DMP 777; Degradation product

1. Introduction

DMP 777, [S-R*,S*]-N[1-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2-[4-[(4-methyl-1-piperazinyl) carbonyl]phenoxy]-4-oxo-1-azetidinecarboxamide is a novel, potent inhibitor of human polymorphonuclear leukocyte elastase which is currently under development in clinical trials for the treatment of cystic fibrosis and rheumatoid arthritis (Fig. 1).

Cystic fibrosis and other inflammatory diseases demonstrate an accumulation of polymorphonuclear leukocytes (neutrophils) at the site of inflammation which release proteinases, such a elastase, which cause damage by attacking susceptible extracellular tissue [1].

DMP 777 is currently formulated as a capsule for oral dosing and has been stored under con-

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PII: S0731-7085(00)00461-1

trolled stability conditions for over 2 years. Stability testing of the product has shown an unexpected degradation product greater than 0.10% at 3 months 40°C/75% r.h. and 2 years 25°C/60% r.h. (Fig. 2).

The current International Conference on Harmonisation (ICH); Guideline On Impurities In New Drug Product, QB3, [2] requires each degradation product greater than 0.2% (mass) to be identified when the daily dose of the drug is between 100 mg and 2 g. Isolation and characterization (identification) of the degradation product in the DMP 777 capsule formulation were conducted because it approaches the 0.2% threshold set forth in the ICH guideline. Early in development when the identity of impurities is unknown or standards do not exist, it is common to use chromatographic peak area to approximate the concentration of impurities.

Dilute acid refluxing was used to hydrolyze the DMP 777 drug substance forming several degradants. Analytical HPLC retention time was used to correlate the peaks generated by refluxing with the degradant formed during stability studies with the capsule formulation. Preparative HPLC was then used to isolate the degradant of interest and it was subsequently characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR). Other known degradants were also isolated and confirmed by LC-MS or analytical HPLC retention time comparison to authentic substance material.

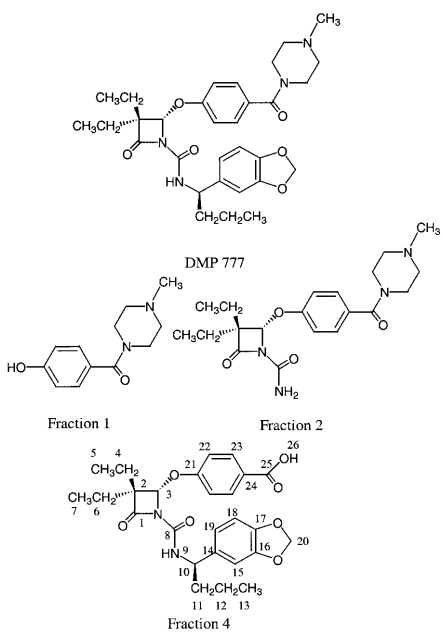

Fig. 1. Chemical structure of DMP 777 and degradation products fraction 1 (F1), fraction 2 (F2), and fraction 4 (F4).

2. Experimental

2.1. Materials

HPLC grade acetonitrile and methanol (EM Science, Gibbstown, NJ), HPLC grade trifluoroacetic acid (Burdick & Jackson, Muskegen, MI), 2-[*N*-morpholino] ethanesulfonic acid monohydrate (Sigma Chemical Company, St. Louis, MO), 2-[*N*-morpholino]ethanesulfonic acid sodium salt (Sigma Chemical Company, St. Louis, MO), HPLC grade sodium octanesulfonate (J.T. Baker, Phillipsburg, NJ) and deionized water from a Milli-Q Plus ultra-pure water system (Millipore, Molsheim, France) were used in the preparation of mobile phases. Reagent grade hydrochloric acid (EM science, Gibbstown, NJ) was used to prepare 0.1 N hydrochloric acid.

2.2. Preparative chromatography

Preparative scale experiments were performed on a preparative HPLC system consisting of a rabbit solvent delivery system (Varian Inc. Walnut Creek, CA), a Gilson model 201 fraction collector (Gilson Medical Electronics, Middleton, WI), and a Knauer variable wavelength monitor (Varian Inc. Walnut Creek, CA) controlled by the Dynamax HPLC Method Manager, version 1.4.6 software (Varian Inc. Walnut Creek, CA).

The preparative HPLC method utilized a Rainin, Dynamax–60A, C18, 10 mm ID × 25 cm, semipreparative HPLC column with a Rainin, Dynamax–60A, C18, guard column (Varian Inc. Walnut Creek, CA) using a binary reverse-phase gradient. Mobile Phase A was water:methanol:trifluoroacetic acid (90:10:0.1). Mobile Phase B was methanol:trifluoroacetic acid

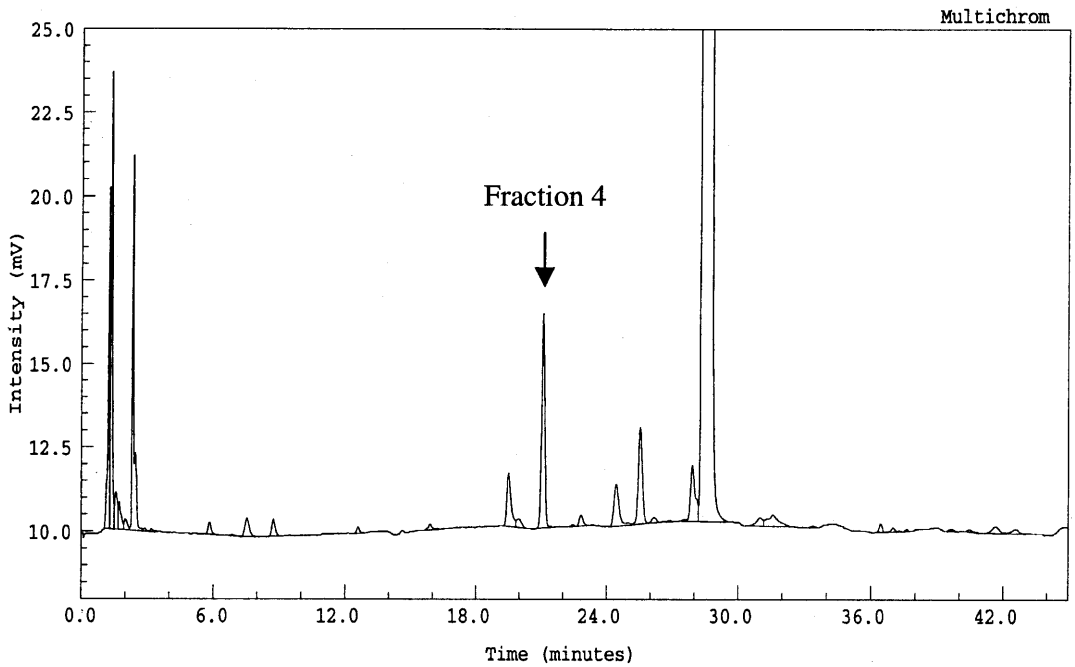

Fig. 2. Typical analytical chromatogram of the DMP 777 capsule formulation stored at 3 months 40°C/75% r.h.

(100:0.1). A linear gradient from 65 to 100% B in 20 min was used for the separation. Detector wavelength was 254 nm, flow rate was 7.0 ml/min, column temperature was ambient and 2 ml of a 40 mg/ml solution was injected.

2.3. Analytical chromatography

Analytical scale experiments were performed on a Waters (Waters, Milford, MA) HPLC system consisting of a 600E multisolvent delivery system, an HPLC column oven (Waters TCM/CHM), a model 486 tunable absorbance detector, and a model 717 autosampler. The chromatographic data was acquired and analyzed on a Multichrom™ data system (Thermal Labsystems, Cheshire, UK).

The analytical HPLC method utilized a Waters (Waters, Milford, MA), Symmetry, C18, 4.6 × 250 mm HPLC column using a binary reverse-phase gradient. Mobile Phase A was acetonitrile:buffer solution:sodium octanesulfonate (250 mM) (30:66:4) where the buffer solution contains 0.1% 2-[*N*-morpholino]ethanesulfonic acid, monohydrate and 0.2% 2-[*N*-morpholino]ethanesulfonic acid, sodium salt. Mobile Phase B was acetonitrile:buffer solution:sodium octanesulfonate (250 mM) (70:26:4) where the buffer solution contains 0.1% 2-[*N*-morpholino]ethanesulfonic acid, monohydrate and 0.2% 2-[*N*-morpholino]ethanesulfonic acid, sodium salt. A linear gradient from 0 to 100% B in 35 min was used for the separation. Detector wavelength was 250 nm, flow rate was 1.5 ml/min, injection volume was 15 μl, and the oven temperature was 50°C.

2.4. Generation of degradation product

The degradant of interest was prepared in quantity by refluxing DMP 777 drug substance in dilute acid. The reflux sample was prepared by dissolving 2 g of DMP 777 drug substance in 10 ml of methanol and then 100 ml of 0.1 N HCl. This solution was refluxed for 32 h and samples were taken periodically to monitor the degradation of the drug substance. The refluxed sample was evaporated to dryness and reconstituted in

methanol to a final concentration of approximately 40 mg/ml.

2.5. Isolation of impurities

The peak of interest (fraction 4) was collected during the entire scale up process with a total of approximately 15 injections made ranging from 15 μl to 2 ml for a final yield of approximately 160 mg. The isolated degradation product was evaporated to dryness in an evaporation dish over several days, and the resulting residue was collected and used for structure elucidation. Samples of the isolated degradation product were examined throughout the evaporation phase by analytical HPLC to ensure that no further degradation occurred.

2.6. Mass spectrometry

Low-resolution electrospray ionization (ESI) mass spectra were obtained on a Micromass Platform II single-quadrupole mass spectrometer (Micromass, Manchester, UK). A 10-μl aliquot of a 5 μg/ml sample solution in water:acetonitrile (1:1) was injected and introduced into the mass spectrometer (without separation) via a Hewlett-Packard 1050 autosampler and HPLC pumping system. The mobile phase was water:acetonitrile (1:1) and the flow rate was 0.25 ml/min. Data were acquired in alternating positive-ion (ES+) and negative-ion (ES−) modes. Ions were generated in ES+ mode under the following conditions: capillary potential, +3.5 kV; sampling cone potential, +25 V. Ions were generated in ES− mode under the following conditions: capillary potential, −3.2 kV; sampling cone potential, +25 V. In both modes, the source temperature was 150°C, the scan range was 150–1000 Da and the scan rate was 1 scan per s.

Low-resolution atmospheric pressure chemical ionization (APcI) mass spectra were obtained on a Finnigan Navigator-aQa single-quadrupole mass spectrometer (ThermoQuest, Manchester, UK). A 10-μl aliquot of a 0.2 mg/ml sample solution in water:acetonitrile (1:1) was injected and introduced into the mass spectrometer (without separation) via a Thermal Separations AS3000 autosampler and P4000 HPLC pumping system. The mobile phase was water:acetonitrile (1:1) and the flow rate was 0.25 ml/min. Data were acquired in alternating positive-ion (AP+) and negative-ion (AP−) modes. Ions were generated in AP+ mode under the following conditions: corona pin potential, +3.5 kV; sampling cone potential, +20 V. Ions were generated in AP− mode Funder the following conditions: corona pin potential, −3.5 kV; sampling cone potential, −20 V. In both modes, the APcI probe temperature was 250°C, the scan range was 150–1000 Da and the scan rate was 1 scan per s.

2.7. Nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance (NMR) data were obtained using a solution of the compound in dmso,d6. Proton and carbon chemical shifts were referenced to the solvent at 2.49 and 39.5 ppm, respectively. The structure assignment was based upon data from the following experiments — 1D proton and carbon spectra, 1D proton nOe difference spectra, 2D proton–proton correlated spectroscopy (GCOSY), 2D proton–carbon single bond correlated spectroscopy (GHSQC) and 2D proton–carbon multiple bond correlated spectroscopy, (GHMBC).

The nOe difference spectra were acquired on a Varian (Palo Alto, CA) Unity-400 NMR spectrometer operating at 399.73 MHz. A saturation time of 30 s was used for the nOe difference experiment. All other spectra were acquired on a Varian Inova-500 NMR spectrometer, with proton detector at 499.91 MHz and with carbon detection at 125.72 MHz. The probe used for these experiments was an inverse detection probe equipped with a z-gradient coil.

3. Results and discussion

3.1. Major degradants in the dilute acid reflux sample

Four major degradants were observed in the chromatogram of the dilute acid reflux sample (Fig. 3 and Fig. 4). Two of the four peaks have

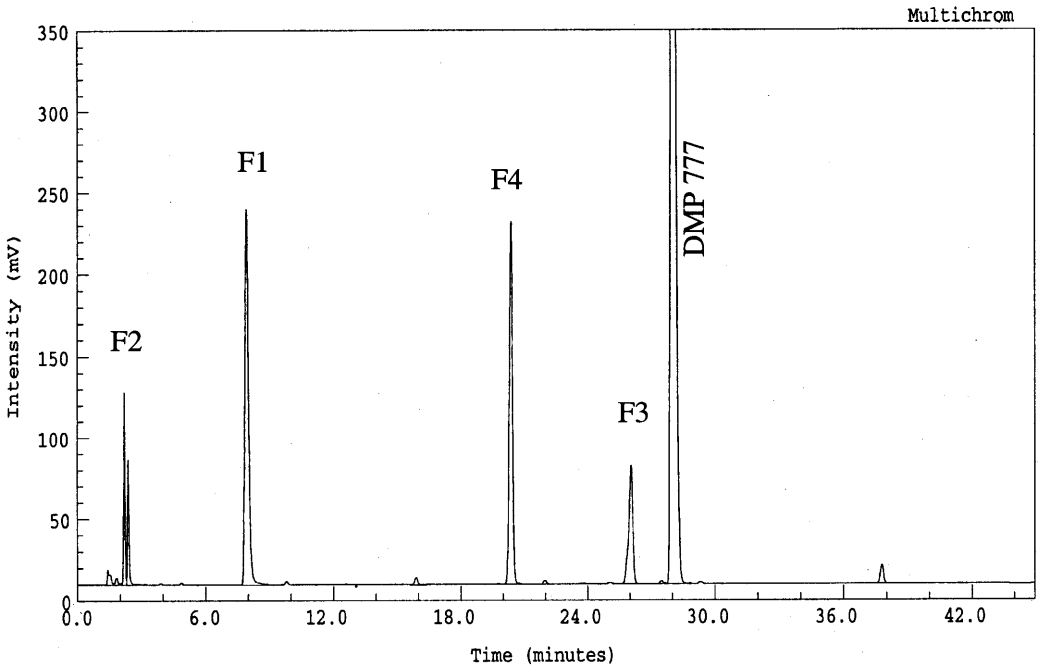

Fig. 3. Analytical chromatogram of the dilute acid reflux sample.

been observed in the capsule formulation and therefore are of the greatest importance. The other two peaks are of lesser importance and will be discussed only briefly.

Fraction 1 (F1) has not been observed in the capsule formulation. Previous work done to demonstrate degradation kinetics of DMP 777 identified several degradation products produced under acid and base conditions [3]. F1 was one of the compounds confirmed by mass spectrometry in that paper and its structure is shown in Fig. 1. Fraction 3 (F3) also has not been observed in the capsule formulation and was not identified. Fraction 2 (F2) corresponds to a previously identified degradation product observed in the capsule formulation and is shown in Fig. 1. This work focused on the identification of Fraction 4 (F4) because it was observed in the capsule formulation and not yet identified. F4 is shown in Fig. 1.

3.2. Isolation of unknown degradant found in capsule formulation

Preparative HPLC is a well-established tech-

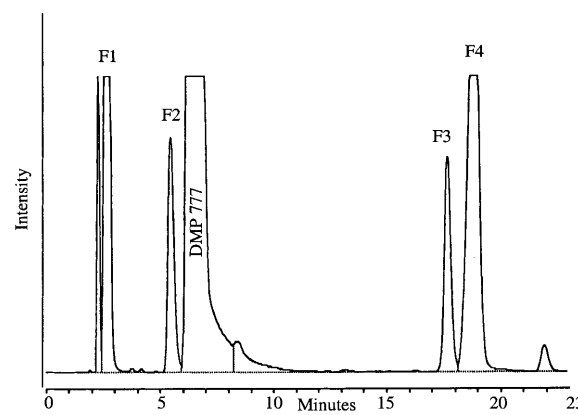

Fig. 4. Preparative chromatogram of the dilute acid reflux sample.

nique for isolating pure compounds from mixtures and was used successfully in this application to separate and isolate the degradant of interest, F4 [4,5]. The degradant observed in the capsule formulation was slightly greater than 0.10% and this was too small a quantity to be easily isolated by preparative HPLC so a larger quantity of this degradant was generated by dilute acid refluxing of DMP 777 drug substance. The amount of degradation in the reflux sample was monitored by analytical reverse phase HPLC. When the peak of interest reached approximately 20% of the total by analytical reverse phase HPLC area percent, the refluxing was stopped. A preparative HPLC method was developed to separate and isolate the degradant of interest. Over the course of the preparative HPLC method development, conditions were continuously modified to improve the separation until scale-up was completed. Fractions were collected during the entire scale up process and at the final preparative HPLC sample loading conditions. The purity of the degradation product isolated by preparative HPLC was greater than 97%, as judged by analytical reverse phase HPLC area percent. The retention time was found to be identical to the retention time of the degradation product found in the capsule formulation.

3.3. Mass spectrometry data interpretation

All of the mass spectral data are consistent with a molecular weight of 482 Da.

The ES+ spectrum is indicative of the presence of a carboxylic acid functionality. The ions observed at m/z 505 are consistent with the $(M+Na)^+$ ion for a free acid of MW 482 and/or $(M+H)^+$ for the analogous sodium acid salt; likewise, the ions observed at m/z 527 are consistent with $(M+Na)^+$ for the sodium acid salt. This pattern is often observed for free carboxylic acids. The ions observed at m/z 546 and m/z 568 correspond to adducts of m/z 505 and m/z 527, respectively, with acetonitrile from the mobile phase. Other observed ions include $(2M+Na)^+$ at m/z 987. The ES− spectrum exhibits $(M-H)^-$ molecular ions at m/z 481 and $(2M-H)^-$ ions at m/z 963.

The AP+ spectrum exhibits $(M+H)^+$ molecular ions at m/z 483. The AP$^-$ spectrum exhibits weak $(M-H)^-$ ions at m/z 481 and $(M+CF_3COO^-)^-$ cluster ions at m/z 595.

3.4. NMR data interpretation

The following proton and carbon NMR peak assignments were based upon 2D correlation spectra and nOe difference spectra. ^{1}H-NMR (dmso,d6) δ 12.71 (br. s, H26), 7.90 (m, 2H, H23), 7.29 (d, $J=8.2$, H9), 7.23 (m, 2H, H22), 6.93 (d, $J=1.6$, H15), 6.83 (d, $J=8.0$, H18), 6.76 (dd, $J=1,6,8,0$, H19), 6.02 (s, H3), 5.97 (br. s, 2H, H20), 4.64 (ddd, $J=7.1$, 8.2, 8.2, H10), 1.79 (m, 4H, H6, H4, H11), 1.65 (m, 2H, H6, or H4, H11).1.19 (m, 2H, H12), 0.94 (t, 3H, $J=7.5$, H5 or H7), 0.87 (t, 3H, $J=7.4$, H7 or H5), 0.84 (t, 3H, $J=7.4$, H13).

^{13}C-NMR (dmso,d6) δ 170.4 (C1), 166.7 (C25), 160.0 (C21), 148.2 (C8), 147.3 (C16), 146.1 (C17), 136.6 (C14), 131.2 (C23), 124.8 (C24), 119.7 (C19), 116.4 (C22), 107.9 (C18), 106.8 (C15), 100.8 (C20), 84.1 (C3), 63.1 (C2), 53.4 (C10), 37.9 (C11), 22.7 (C4 or C6), 20.6 (C6 or C4), 19.0 (C12), 13.4 (C13), 8.6 (C5 or C7), 8.2 (C7 or C5).

4. Conclusion

Low level degradation products can have an impact on the safety of a pharmaceutical dosage form. In situations where there is a small but significant amount of degradation in a drug or drug product, it can be difficult to isolate the peak of interest for proper structure elucidation. In some situations, the degradation products may be created in larger quantities by stressing the drug product or the drug substance itself. This work utilized acid hydrolysis to produce larger amounts of the degradation product, facilitating preparative isolation. The drug degradation product was found to be identical to that observed in the drug product stability samples by retention on analytical HPLC and the expected chemistry of the parent molecule. The use of mass spectrometry and 2D NMR spectroscopy resulted in the unambiguous assignment of structure for the degradation product found in this DMP 777 capsule formulation.

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