Department of Drug Metabolism[1], Merck Research Laboratories, West Point, PA, USA and Chemistry Department[2], Lehigh University, Bethlehem, PA, USA

Isolation and structural characterization of the photolysis products of etoricoxib

C. Z. Matthews[1], R. Subramanian[1], E. J. Woolf[1], N. Foster[2], B. K. Matuszewski[1]

Received March 9, 2004, accepted March 15, 2004

Catherine Z. Matthews, Department of Drug Metabolism, Merck Research Laboratories, WP 26-372, West Point, PA, USA
catherine_matthews@merck.com

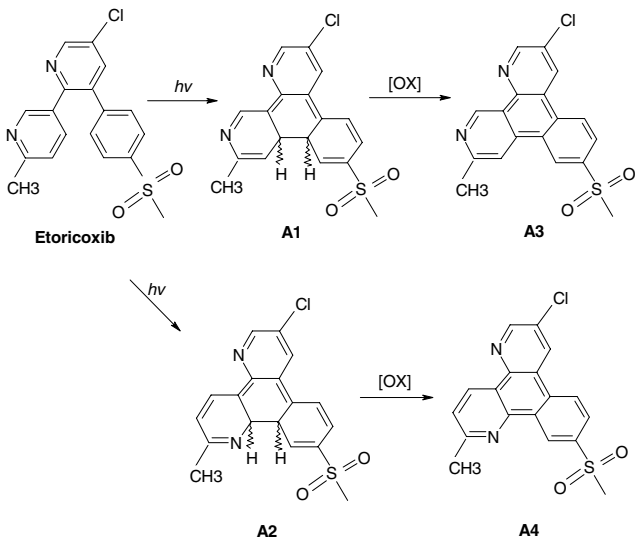
Pharmazie 59: 913–919 (2004)

Etoricoxib is a potent and novel selective inhibitor of cyclooxygenase-2 (COX-2) which has been developed for the treatment of osteoarthritis, rheumatoid arthritis and several other inflammatory conditions. To support clinical pharmacokinetics studies, a method for the determination of etoricoxib in human plasma was developed. During the development of the method it was found that highly fluorescent products were formed when etoricoxib was exposed to UV light (254 nm). The formation of highly fluorescent products was the basis for the development of a highly sensitive HPLC/fluorescent assay for the indirect determination of etoricoxib in human plasma; the limit of quantification (LOQ) was 1 ng/mL. To unequivocally determine the chemical structures of the photolysis products of etoricoxib, a series of studies was conducted. When etoricoxib was irradiated online in a photochemical reactor, three products were detected in an HPLC-UV system. These products were characterized by HPLC-UV-fluorescence and HPLC-MS/MS. Possible structures of these products were proposed based on these data. The major photolysis products of etoricoxib were further isolated and their structures were elucidated using NMR and HPLC-NMR. The results of these experiments indicate that etoricoxib undergoes a photocyclization reaction when irradiated with UV light (254 nm), leading to the formation of two major isomeric photocyclization products.

1. Introduction

Etoricoxib (5-chloro-3-(4-methanesulfonylphenyl)-6′-methyl-[2,3′]bipyridinyl, Scheme) is a potent and novel selective second generation COX-2 inhibitor. Its anti-inflammatory properties used for the treatment of osteoarthritis, rheumatoid arthritis, and pain have been demonstrated in human clinical studies (Gottesdiener et al. 2002; Matsumoto et al. 2002; Cochrane et al. 2002). During the clinical development program for etoricoxib, a highly sensitive analytical method (limit of quantitation (LOQ) is 1 ng/mL) for the determination of etoricoxib in human plasma was required. A method based on HPLC with fluorescence detection after post-column photochemical derivatization was developed (Matthews et al. 2001). It was found that the high sensitivity and selectivity of the assay resulted from etoricoxib forming highly fluorescent products, under acidic conditions, after exposure to UV light at 254 nm. A possible series of reactions that result in the formation of these products has been proposed and is shown in the Scheme. The proposed reaction scheme is analogus to the cyclization of rofecoxib, which is known to undergo a stilbene-phenanthrene type photocyclization when irradiated with UV light (254 nm) (Woolf et al. 1999). To elucidate and confirm the formation of photolysis products of etoricoxib, a series of photolysis experiments was conducted, the products of photolysis were isolated and their chemical structures were confirmed using HPLC-NMR, HPLC-MS/MS and other spectroscopic techniques. The results of these studies are subject of this publication.

Scheme



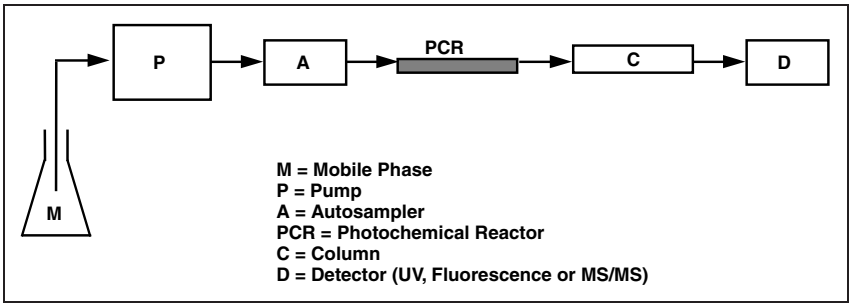


Fig. 1:
A schematic representation of an online irradiation system

2. Investigations, results and discussion

2.1. Online photochemical studies

When etoricoxib was injected into the HPLC-UV/fluorescence system (Fig. 1) with the photochemical reactor lamp off, a single peak with a retention time of 2.6 min was observed (Fig. 2A). The UV spectrum of etoricoxib in the region above 200 nm contains two absorption bands with maxima at 238 nm and 280 nm (Fig. 3C). When etoricoxib was injected into the same system with the reactor lamp on, three new photolysis products were detected with retention times of 4.7 (Peak 1), 7.0 (Peak 2) and 19.6 (Peak 3) minutes; unreacted etoricoxib ($t_r = 2.6$ min) was practically not detected (Fig. 2B). The UV spectra of the photolysis products from Peak 1 and Peak 3 were very similar and the absorption maxima were shifted to longer wavelengths, compared to etoricoxib. Only one absorption maximum at 258 nm (Fig. 3A and B) was observed. These photolysis products from Peak 1 and Peak 3 could also be detected via their fluorescence. The photolysis product from Peak 2 had a different UV spectrum (Fig. 3D), compared with the photolysis products from Peak 1 and Peak 3. This product was not fluorescent and its UV absorption maximum was shifted to longer wavelengths compared to etoricoxib. It had three absorption bands with maxima at 250 nm, 285 nm and 330 nm (Fig. 3D). These results could be explained, based on the previously proposed photocyclization scheme. The photolysis products from Peak 1 or Peak 3 (Fig. 2B) may correspond to **A3** or **A4** as these fully conjugated products would be expected to be fluorescent. The large difference in retention time of **A3** and **A4** may be due to differences in their pKa values. The product from Peak 2 (Fig. 2B) may correspond to **A1** or **A2**, as these products would be expected to exhibit significantly lower fluorescence.

Data from the online HPLC-MS/MS analysis provided some structural information on the photolysis products. Mass spectra of the photolysis products corresponding to Peak 1 and Peak 3 (Fig. 2B) both exhibited the presence of a protonated molecule at m/z 357. The photolysis product from Peak 2 (Fig. 2B) was characterized by the presence of a protonated molecule at m/z 359. When these ions at m/z

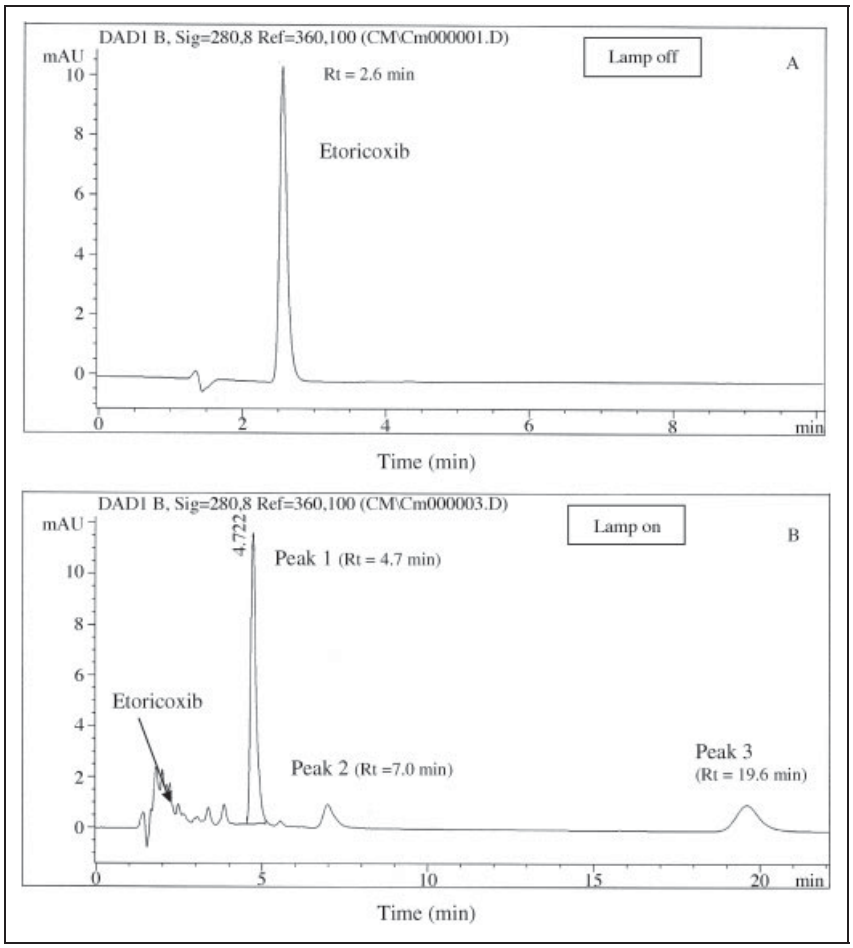


Fig. 2:
HPLC-UV chromatograms of etoricoxib subjected to online irradiation in HPLC-UV-Fluorescence system, with photochemical reactor's lamp on or off

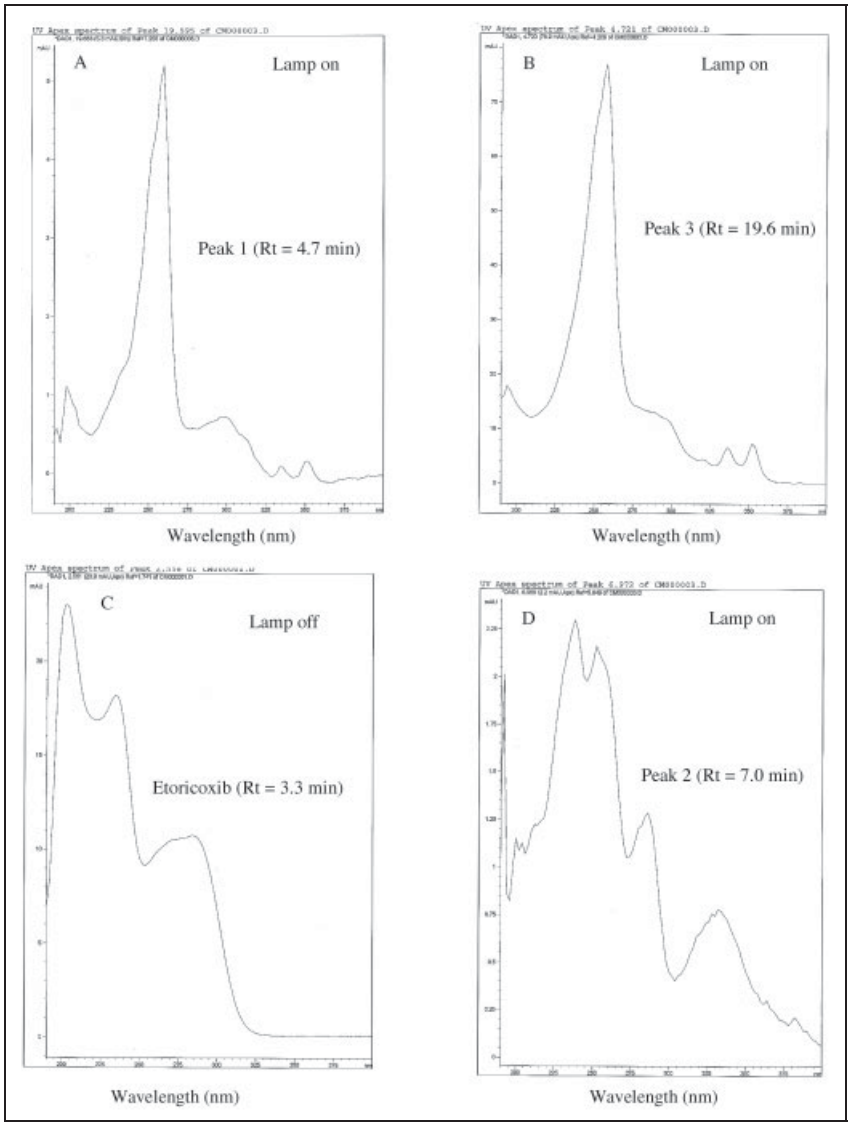


Fig. 3:
UV Spectra of etoricoxib, and products corresponding to Peak 1, Peak 2 and Peak 3 when etoricoxib was subjected to online irradiation in the HPLC-UV-fluorescence system, with photochemical reactor's lamp on or off

357 and m/z 359 were subjected to collision induced fragmentation, major product ions at m/z 278 and m/z 280 were observed. These product ions correspond to the loss of the methyl sulfonyl group from the proposed products **A1**–**A4**.

2.2. Photolysis product isolation

Confirmation of the proposed chemical structures of the products corresponding to Peaks 1, 2 and 3 required NMR or HPLC-NMR analysis. In order to obtain the NMR spectra, sufficient amounts of the compounds from Peak 1, 2 and 3 had to be isolated. However, when etoricoxib was irradiated off-line for 1 min, Peak 2 was not detected under the same chromatographic conditions as those used when etoricoxib was irradiated on-line, probably due to highly efficient oxidation from **A1/A2** to **A3** and /or **A4**. It was therefore impossible to isolate the compound corresponding to Peak 2 under the experimental conditions employed. Peaks 1 and 3 were found to be formed during off-line irradiation, and their isolation for NMR analysis was attempted.

The effect of irradiation time on product yield was studied in order to obtain the highest yield of these products. Etoricoxib solutions at a fixed concentration of 8 μg/mL, were irradiated for different periods of time. Separate portions of the solution (n = 10) were exposed to UV light in the off-line photochemical reactor for time periods of 5 s, 15 s, 30 s, 1, 2, 3, 4, 5, 6, 8, and 12 min. The irradiated samples were then transferred into amber autosampler vials and injected onto the HPLC-UV/fluorescence system. The peak areas of Peak 1 and Peak 3 were plotted against irradiation time (Fig. 4). The data indicated that photolysis product corresponding to Peak 1, referred to as compound **A,** reached its highest concentration when the solution was irradiated for 1 min, while for photolysis product corresponding to Peak 3, compound **B**, the maximum concentration was observed between 1 and 2 min. Therefore a 1-min irradiation time was chosen for the preparative photolysis procedures. With longer irradiation times, compounds **A**

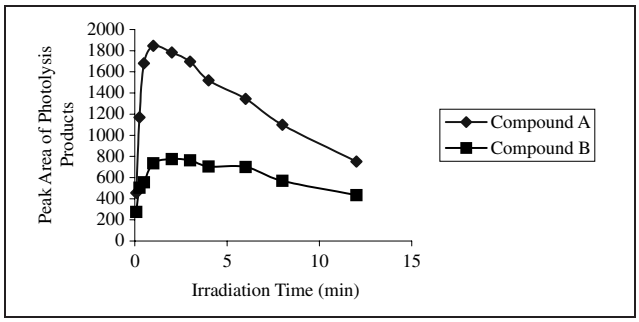


Fig. 4: The formation of compounds **A** and **B** when a fixed concentration of etoricoxib was irradiated off-line for different periods of time

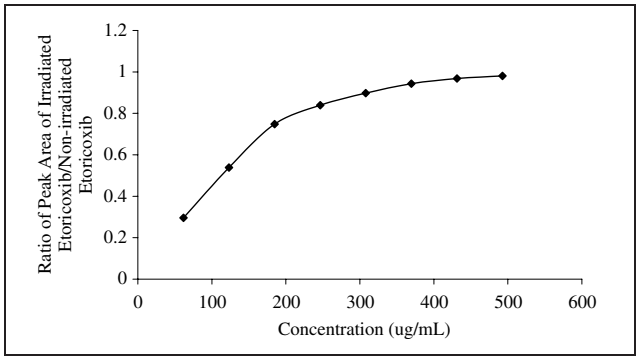


Fig. 5: The ratio of peak areas of irradiated etoricoxib and non-irradiated etoricoxib at different etoricoxib concentrations; irradiation time of one minute

and **B** were converted into other secondary photolysis products which were not the subject of this investigation.

To maximize the production of primary photolysis products during irradiation, the parent compound should be present in excess in solution; only a fraction (less than 30–50%) of parent compound should be converted to photolysis products leaving the majority of the parent compound unchanged. For etoricoxib, the goal was to convert less than 10% of the compound to photolysis products to avoid formation of any secondary photolysis products. Solutions of etoricoxib with concentrations ranging from 62 μg/mL to 490 μg/mL were prepared; two samples were prepared at each concentration. At each concentration one sample was irradiated for 1 min while the other was not irradiated. Irradiated samples and non-irradiated samples at each concentration were transferred into amber autosampler vials and injected onto the HPLC-UV/fluorescence system (Fig. 1, without the photochemical reactor). The ratios of areas of the etoricoxib peaks with and without irradiation at each concentration were plotted against the solution concentration as shown in Fig. 5. The results indicated that one minute irradiation of a solution containing etoricoxib at the concentration greater than 400 μg/mL resulted in the conversion of less than 10% of the com-

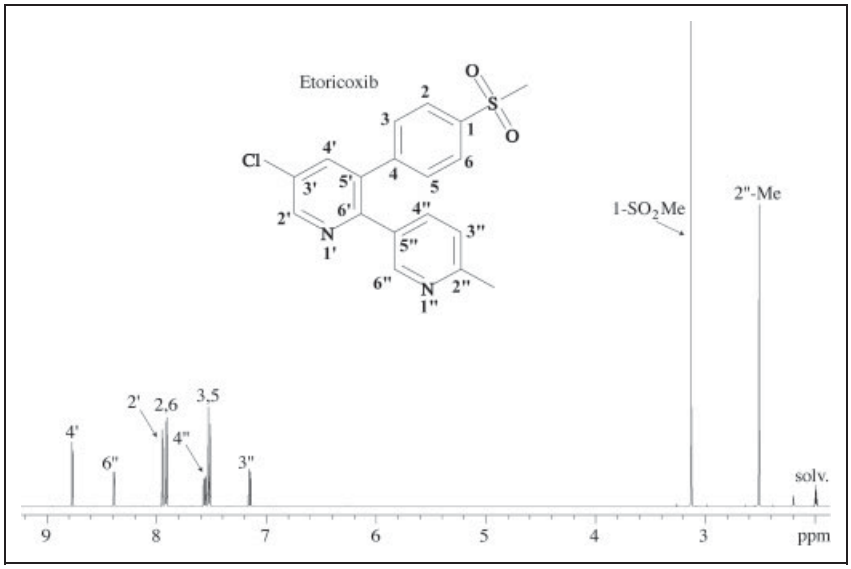


Fig. 6:
1D ^{1}H spectrum of etoricoxib in CD_3CN

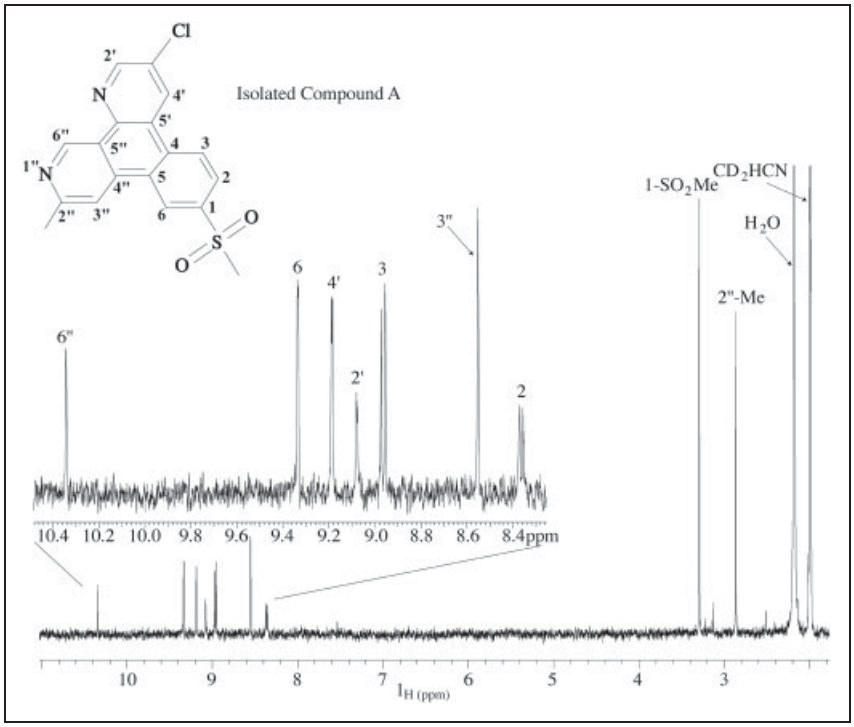


Fig. 7:
1D ^{1}H spectrum of compound **A** in CD_3CN

pound to photolysis products, hence insuring that primary photolysis products were formed predominately during irradiation. After selecting the optimal irradiation time and concentration conditions, compounds **A** and **B** from Peak 1 and Peak 3 were readily isolated in the solid form for further characterization.

2.3. Structure elucidation of compounds A and B

A 1D ^{1}H spectrum of etoricoxib in CD_3CN is shown in Fig. 6. The methyl sulfone and 2″-Me appear at 3.13 and 2.51 ppm, respectively. Protons 2/6 and 3/5 of the methyl sulfone phenyl ring appear at 7.91 and 7.56 ppm respectively with J = 8.4 Hz. Protons 2′ and 4′ of the chloro pyridine ring appear at 7.95 and 8.77 ppm respectively. Finally, protons 3″, 4″, 6″ of methyl pyridine appear at 7.15, 7.56, and 8.39 ppm respectively, with a scalar coupling pattern expected from a 2, 5 substituted pyridine ring (Table).

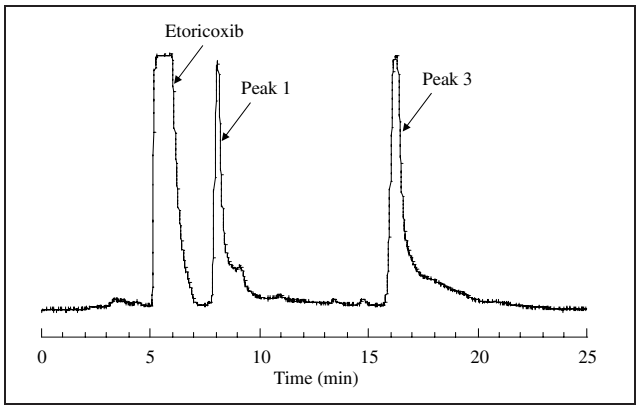


Fig. 8: HPLC-UV Profile at 258 nm for Peak 1 and Peak 3 in HPLC-NMR Analysis

Isolated compound **A** (Peak 1) was dissolved in CD_3CN. 1D ^{1}H, 2 D Total Correlation Spectroscopy (TOCSY) and ROESY data sets were acquired at 25 °C. Relative to the parent 1D spectrum, all aromatic region protons in compound **A** spectrum were shifted substantially downfield (Table), consistent with the proposed ring closure aromatic structures of **A3** or **A4**. The following through bond correlation are observed in the TOCSY spectrum: 9.08 (H2′) and 9.19 ppm (H4′); 2.86 (2″-Me), 8.56 (H3″), and 10.35 ppm (H6″); 8.37 (H2), 8.97 (H3), and 9.33 (H6). ROESY cross peaks are observed from H3 of the methylsulfone-phenyl ring to H2 and H4′ of chloro-pyridine ring. An ROE cross peak is also seen from H6 of the methylsulfone-phenyl ring and H3″ of methyl pyridine ring. The combined NMR data agree with the structure of **A3**, indicating that the ring closure has occurred across the C4″–C5 bond followed by aromatization as shown in the Scheme.

The structure of compound **B** was elucidated using HPLC-NMR because the amount of compound **B** isolated was not enough to conduct conventional NMR analysis. The HPLC profile for the HPLC-NMR run is shown in Fig. 8. The 1D ^{1}H, gCOSY and ROESY data were collected on the parked analyte peak. All aromatic region protons in the spectrum of compound **B** are shifted substantially downfield compared with the parent compound (Table and Fig. 9), consistent with the proposed ring closure scheme. Anticipated COSY cross peaks are seen between H4′ and H2′ of the chloro-pyridine ring, H3″ and H4″ of the methyl pyridine ring, and H2 and H3 of the methylsulfone-phenyl ring. However, ROE cross peaks are only seen between H3 and H4′. Hence, the combined

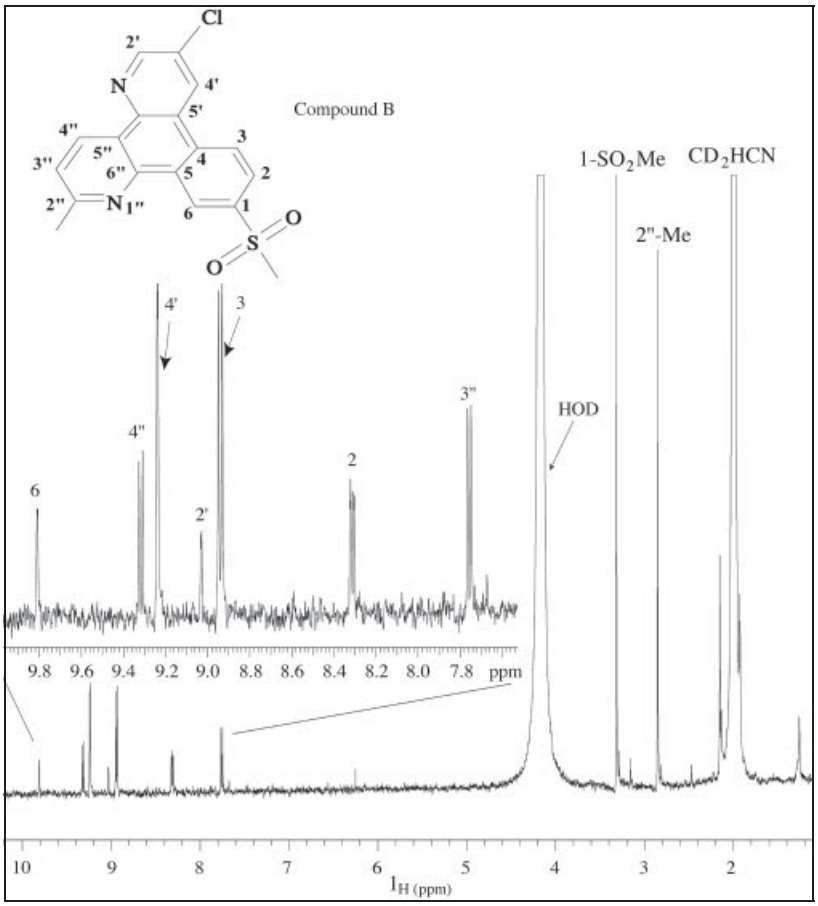


Fig. 9:
1D ^{1}H spectrum of compound **B** in CD_3CN

Table: Identifiable proton chemical shifts for etoricoxib, compound A, and compound B

	Etoricoxib	Compound **A**	Compound **B**
1-SO_2Me	3.13 (s)	3.29 (s)	3.32 (s)
2	7.91 (d, J = 8.4 Hz)	8.37 (dd, J = 8.8, 1.8 Hz)	8.31(dd, J = 8.8, 1.8 Hz)
3	7.52 (d, J = 8.4 Hz)	8.97 (d, J = 8.8 Hz)	8.94 (d, J = 8.6 Hz)
5	7.52 (d, J = 8.4 Hz)	–	–
6	7.91 (d, J = 8.4 Hz)	9.33 (d, J = 1.8 Hz)	9.81 (d, J = 1.8 Hz)
2′	7.95 (d, J = 2.4 Hz)	9.08 (d, J = 2.2 Hz)	9.03 (d, J = 1.8 Hz)
4′	8.77 (d, J = 2.4 Hz)	9.19 (d, J = 2.2 Hz)	9.24 (d, J = 1.8 Hz)
2″-Me	2.51 (s)	2.86 (s)	2.85 (s)
3″	7.15 (d, J = 8.2 Hz)	8.56 (s)	7.76 (d, J = 8.3 Hz)
4″	7.56 (dd, J = 2.4, 8.2 Hz)	–	9.32 (d, J = 8.3 Hz)
6″	8.39 (d, J = 2.4 Hz)	10.35 (s)	–

Note: d = doublet, dd = double doublet, s = singlet, J = Scalar coupling constant
All shifts are referenced to CD_2HCN multiplet set at 1.99 ppm (See Fig. 6 and 7 for chemical structures and numbering of carbon atoms)

NMR data supports the proposed structure for **A4**, indicating that the ring closure has occurred across the C6″–C5 bond followed by aromatization to yield **A4** as proposed in the Scheme.

2.4. UV and mass spectra of isolated compounds A and B

The isolated solid compounds **A** and **B** were reconstituted in mobile phase and their UV absorption spectra and mass spectra were obtained. The UV spectra of compounds **A** and **B** were found to be similar to these observed for Peak 1 and Peak 3 (Fig. 3) recorded during online irradiation. The mass spectra obtained from isolated compounds **A** and **B** were also similar to these obtained in the online irradiation experiments. These data suggest that the isolated compounds are the same as those formed during online irradiation.

2.5. Fluorescence quantum efficiencies of compounds A and B

The fluorescence quantum efficiencies (Φ_f) for compounds **A** and **B** were determined relative to triphenylene as a fluorescence standard. Triphenylene exhibits a fluorescence and a UV absorption spectra that are similar to those of compounds **A** and **B** (Murov et al. 1993). The following equation was used for fluorescence quantum effeciency calculation of compounds **A** and **B** (Matuszewski et al. 1987):

$$\Phi_f/\Phi_{f'} = I_f(1 - 10^{-A'})/[I_{f'}(1 - 10^{-A})]$$

where: Φ_f = fluorescence quantum efficiency of the photolysis products (compounds **A** and/or **B**) $\Phi_{f'}$ = fluorescence quantum efficiency of triphenylene ($\Phi_{f'} = 0.09$ in ethanol) (Murov et al. 1993) I_f = measured fluorescence intensity of the photolysis products, $I_{f'}$ = measured fluorescence intensity of triphenylene A = absorbance of the photolysis products at the wavelength of excitation (258 nm) A′ = absorbance of triphenylene at 258 nm.

The concentrations of triphenylene, and compounds **A** and **B** in ethanol were adjusted such that their UV absorbances (A and A′) at the wavelength of excitation (258 nm) were close to 0.1. Fluorescent intensities (I_f and $I_{f'}$) of the solutions were then measured (Matuszewski et al. 1987). Fluorescence quantum effeciencies of compounds **A** and **B** were found to be 0.34 and 0.41, respectively.

In conclusion, data obtained in these experiments support the assignment that photolysis products designated compounds **A** and **B** correspond to **A3** and **A4,** respectively Scheme. Overall these data indicate that etoricoxib undergoes a photocyclization reaction analogous to other stilbene-phenanthrene light generated electrocyclic reactions. The relatively high fluorescence quantum efficiencies of **A3** and **A4** (Φ_f of A3 = 0.34, Φ_f of A4 = 0.41) indicate that both of these photolysis products of etoricoxib are highly fluorescent compounds, which are likely responsible for highly sensitive nature of previously described (Matthews et al. 2001) procedure for the determination of etoricoxib in human plasma.

3. Experimental

3.1. Materials

Etoricoxib was provided by Merck-Frosst (Montreal, Canada). Triphenylene was purchased from Aldrich-Sigma (Milwaukee, WI). Acetonitrile (ACN, HPLC grade), methanol (Omnisolve HPLC grade) and ethanol were purchased from EM Science (Gibbstown, NJ). All other reagents were ACS grade from Fisher Scientific (Fair Lawn, NJ). Water was purified on a Milli-Q system and had a resistivity of 18.2 Ω at the outlet. Deuterated acetonitrile (CD_3CN, 99.8% D), deuterated water (D_2O, 99.9% D), deuterated ammonium hydroxide (ND_4OD, 99% D) and deuterated acetic acid (CD_3COOD, 99%D) were purchased from Cambridge Isotope Laboratories (Andover, MA)

3.2. Instrumentation

An HPLC system with an online photochemical reactor and ultraviolet absorption (UV) and fluorescence detectors was used for the photochemical studies. This system consisted of an HP (Hewlett-Packard, Palo Alto, CA) series 1100 LC pump and automatic injector, followed by an ASTEC (Whippany, NJ) Beam Boost photochemical reactor unit consisting of a 254-nm UV lamp mounted with a 5 m, 0.3 mm ID PTFE reaction coil capable of withstanding high (> 1000 p.s.i.) pressure, an HP series 1100 UV diode array spectrophotometer (UV-DAD) and a Perkin-Elmer (Norwalk, CT) LC 240 fluorescence detector. The photochemical reactor was installed *before* the analytical column as shown in Fig. 1 so that etoricoxib was irradiated and then separated from its photolysis products on the column. This was a different arrangement from that used in the clinical assay, where the photochemical reactor was placed *after* the analytical column (Matthews et al. 2001). The detector output was connected to a PE-Nelson (Cupertino, CA, USA) Turbo-Chrom data system via a PE-Nelson 941 analog-to-digital interface. When irradiation of etoricoxib was performed off-line, the same system was used for analysis but without the photochemical reactor.

The HPLC-MS/MS system used for characterizing the photolysis products online and off-line consisted of a Perkin-Elmer (Norwalk, CT) Model 410 pump, a Varian (Varian, Walnut Creek, CA) Prostar® 96 well format autosampler, an ASTEC (Whippany, NJ) Beam Boost photochemical reactor unit as described above, and a PE-Sciex API 3000 triple quadripole mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer atmospheric pressure chemical ionization interface. The mass spectrometer was operated in the positive ionization mode at unit mass resolution. The temperature of the heated nebulizer was set at 350 °C and the nebulizer pressure was set at 90 p.s.i. The sampling orifice was set at 75V. Product ion mass spectra were obtained at a collision energy of 30 V.

An HPLC-UV-fraction collector system was used for isolation of the major photolysis products of etoricoxib. The system consisted of a Perkin-Elmer (Norwalk, CT) Model 410 pump, a Waters (Milford, MA) WISP 717 autosampler, a Perkin-Elmer (Applied Biosystem division, Foster City, CA)

785A UV/VIS detector and an ISCO (Lincoln, Nebraska) Foxy 200™ X-Y fraction collector. The outputs of the detector and fraction collector were connected to a PE-Nelson (Cupertino, CA) Turbo-Chrom data system via two different PE-Nelson 941 analog-to-digital interfaces.
Off-line irradiation of etoricoxib was performed using a "merry-go-around" photochemical reactor (Rayonet, Hamden, CT) equipped with four 254-nm mercury lamps.
The UV absorption and fluorescence spectra (off-line) were obtained using a diode array spectrophotometer (HP 8452, Hewlett-Packard, Palo Alto, CA) and an Hitachi (Danbury, CT) Model F-4500 spectrophotometer, respectively.
The NMR spectra were acquired on a 500 MHz spectrometer (Inova™ system, Varian Inc., Palo Alto, CA) equipped with a 3 mm inverse detection probe (MIDG-3, Nalorac Inc., Martinez, CA). The ^{1}H NMR chemical shifts (in ppm) are referenced relative to the solvent CD_2HCN signal, which was set to 1.99 ppm.
The HPLC system for HPLC-NMR consisted of a HPLC pump (Model 9012, Varian Inc., Walnut Creek, CA) with a manual injector and a photodiode array detector (PDA) (Model 9065, Varian Inc. Walnut Creek, CA). Once the apex of an HPLC peak was detected by the PDA, the pump was stopped after a pre-calibrated delay time (58 s at 0.3 mL/min flow rate). The NMR data were collected using a flow probe equipped with a 60 μL interchangeable flow cell (IFC Probe, Varian Inc.)

3.3. Evaluation of the photolysis products of etoricoxib using an online photochemical reactor with HPLC-UV/fluorescence and HPLC-MS/MS detection

3.3.1. HPLC-UV/fluorescence studies

One mL of etoricoxib solution (20 μg/mL) in 25/75 acetonitrile/10 mM ammonium acetate (pH 4) (v/v,%) was placed in an amber autosampler vial. Aliquots (100 μL) of the etoricoxib solution were injected into the HPLC-UV-fluorescence system (Fig. 1) with the reactor lamp either on or off. The mobile phase consisted of 35/65 (v/v,%) acetonitrile/10 mM ammonium acetate buffer (pH 4) and was pumped through a Hypersil HYPURITY®-C18 column (50 × 3 mm, 5 μm) at a flow rate of 0.6 mL/min.

3.3.2. HPLC-MS/MS studies

A 20 μL aliquot of 2 μg/mL etoricoxib solution in 25/75 acetonitrile/10 mM ammonium acetate (pH 4) (v/v,%) was injected onto an HPLC-MS/MS system with the photochemical reactor lamp either off or on. The same HPLC conditions (mobile phase, column and flow rate) as used in HPLC-UV/fluorescence system were employed. The mass spectra and product ion spectra of etoricoxib and its photolysis products were obtained.

3.4. Off-line irradiation of etoricoxib

One milliliter of etoricoxib solution (20 μg/mL) in 25/75 (v/v,%) acetonitrile/10 mM ammonium acetate (pH 4) was transferred to a 1 mL quartz cuvette. The cuvette was placed in an off-line "merry-go-around" photochemical reactor and irradiated for 1 min. A 100 μL aliquot of the irradiated etoricoxib solution was then injected into the HPLC system described previously with the photochemical reactor lamp off for analysis.

3.5. Isolation of major photolysis products

Etoricoxib stock solution (3 mL of a 1.2 mg/mL solution in 50/50 acetonitrile/water) was diluted with 5 mL of 10 mM ammonium acetate, pH 4; the final concentration was ~500 μg/mL of etoricoxib). This solution (8 mL) was divided into four aliquots of 2 mL each. Each aliquot was transferred into a 3-mL cuvette (Fisher Scientific, Fair Lawn). The cuvettes were placed in a "merry-go-around" photochemical reactor and irradiated for 1 min. After irradiation all solutions were combined, vortexed and transferred into autosampler vials (750 μL). A 150 μL aliquot of the irradiated solution was injected into the HPLC-UV-fraction collector system. Isocratic HPLC conditions were employed. The mobile phase, column and flow rate were similar to the conditions that were used in the online studies described above, except the length of column was increased from 5 cm to 25 cm so that a stronger mobile phase (65/35 (v/v,%) acetonitrile/10 mM ammonium acetate buffer (pH 4)) could be used. With the higher organic content in mobile phase, the elution order of the major photolysis products was not affected but the process of evaporation of collected fractions was facilitated. Five replicate injections were made from each autosampler vial. All eluents were collected using the fraction collector, which was set at a 15 s collection interval. The UV chromatogram and fraction collector tube-change profile were overlaid to locate the tubes containing the major photolysis products. These tubes were placed in a Turbo-Vap evaporator (Zymark, Hopkinton, MA) set at 20 °C for 1 h, and the organic solvent was evaporated under a stream of nitrogen. The remaining aqueous portions were placed in SpeedVac (Forma Scientific, Marietta, OH) overnight or until the samples were dry. The entire isolation procedure was repeated to obtain sufficient quantities of products for structure elucidation.

3.6. NMR and HPLC-NMR analysis

Etoricoxib and one of its isolated photolysis products (Peak 1, Fig. 2B, compound **A**) were dissolved in 160 μL of CD_3CN and transferred to a 3 mm tube for the analysis by NMR.
The HPLC-NMR technique was used to examine another photolysis product (Peak 3, Fig. 2 B, compound **B**). Etoricoxib solution (400 μL) in 50/50 acetonitrile/water at a concentration of 7 mg/mL was diluted with 2 mL of 10 mM ammonium acetate (pH 4) to yield a final concentration of ~1 mg/mL for etoricoxib. This solution (2.4 mL) was transferred into a 3-mL cuvette. The cuvette was placed in an off-line "merry-go-around" photochemical reactor for 1 min. A Thermo Hypersil Hypurity C18 (2 × 150 mm, 5 μm) analytical column was used to separate etoricoxib and its photolysis products in the HPLC system. A mobile phase gradient was delivered through the column at a flow rate of 0.3 mL/min. Mobile phase A was 10/90 (v/v,%) CD_3CN/10 mM ND_4COOCD_3, pD ~4.93 and mobile phase B was 90/10 (v/v,%) CD_3CN/D_2O; the gradient profile was 0-3 min 40% B, 3-28 min 40-90% B, 28.1-32 min 90% B, and 32.1 min 40% B. Once the apex of Peak 3 was detected at a retention time of 16.5 min, the HPLC pump was stopped after a pre-calibrated delay time (58 s) at the end of which compound **B** from Peak 3 was parked in the observed volume of the probe. A 250 μL aliquot of the irradiated solution was manually injected into the HPLC-NMR system to obtain 1D ^{1}H spectrum while a 500 μL aliquot of the irradiated solution was injected for 2D Gradient Correlation Spectroscopy (gCOSY) and 2D Rotational Overhauser Enhancement Spectroscopy (ROESY) analysis.

3.7. UV, fluorescence, and mass spectra of isolated compounds A and B

Isolated compounds **A** and **B** were dissolved in the mobile phase used for the online experiments. The solution was transferred into a 1 mL cuvette. The cuvette was placed in an UV diode array spectrophotometer to obtain UV spectra and a fluorescence spectrophotometer for fluorescence spectra, respectively.
For MS/MS spectra, the isolated compounds **A** and **B** (as solid materials) were dissolved in 400 μL of acetonitrile. An aliquot of the solution (100 μL) was diluted with 10 mM ammonium acetate (pH 4) to 250 μL. The solution (25 μL) was injected into the HPLC-MS/MS system.

References

Cochrane D, Jarvis B, Keating G (2001) Etoricoxib. Drugs 62: 2637–2651.

Gottesdiener K, Schnitzer T, Fisher C, Bockow B, Markenson J, Ko A, DeTora L, Curtis S, Geissler L, Gertz B (2002) Results of a randomized, dose-ranging trial of etoricoxib in patients with osteoarthritis. Rheumatology 41: 1052–1061.

Matsumoto A, Melian A, Mandel D, McIlwain H, Borenstein D, Zhao P, Lines C, Gertz B, Curtis S (2002) A randomized, controlled, clinical trial of etoricoxib in the treatment of rheumatoid arthritis. J Rheumatol 29: 1623–1630.

Matthews C, Woolf E, Lin L, Fang W, Hsieh J, Ha S, Simpson R, Matuszewski B (2001) High-throughput, semi-automated determination of a cyclooxygenase II inhibitor in human plasma and urine using solid-phase extraction in the 96-well format and high-performance liquid chromatography with post-column photochemical derivatization-fluorescence detection. J Chromatogr B 751: 237–246.

Matuszewski B, Given R, Srinivasachar K, Carlson R, Higuchi T (1987) N-substituted 1-cyanobenz[*f*]isoindole: evaluation of fluorescence efficiencies of a new fluorogenic label for primary amines and amino acids. Anal Chem 59: 1102–1105.

Murov S, Carmichael I, Hug G (1993) Handbook of Photochemistry, 2nd Ed., Marcel Dekker, Inc. New York, New York, p50.

Woolf E, Fu I, Matuszewski B (1999) Determination of rofecoxib, a cyclooxygenase-2 specific inhibitor, in human plasma using high-performance liquid chromatography with post-column photochemical derivatization and fluorescence detection. J Chromatogr B 730: 221–227.