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Determination of rofecoxib, a cyclooxygenase-2 specific inhibitor, in human plasma using high-performance liquid chromatography with post-column photochemical derivatization and fluorescence detection

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Abstract

A method for the determination of rofecoxib in human plasma is described. After the addition of an internal standard, buffered (pH 5) plasma samples are extracted with hexane–methylene chloride (50:50, v/v). The extracts are evaporated to dryness and reconstituted in mobile phase. Upon exposure to UV light, the analyte was found to undergo a stilbene–phenanthrene-like photocyclization reaction with the resulting formation of a highly fluorescent species. Thus, the plasma extracts were analyzed via HPLC with post-column photochemical derivatization and fluorescence detection. The assay has been validated in the concentration range of 0.5–100 ng/ml using 1-ml samples. The method has been successfully utilized to support human clinical pharmacokinetic studies.

Keywords: Rofecoxib

1. Introduction

The enzyme cyclooxygenase (COX) is involved in the synthesis of prostaglandins in man. Prostaglandins are known to have a cytoprotective function in the gastrointestinal mucosa as well as to play a role in the body's response to inflammation. Recent work has shown that there are two isoforms of cyclooxygenase in man, COX-1 and COX-2 [1]. COX-1 is found in most tissues throughout the body and is believed to be involved with maintaining homeostasis. COX-2 is present in high levels at sites of inflammation.

Currently marketed non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit both COX-1 and COX-2 [2]. Thus, while NSAIDs relieve symptoms of inflammation, they also inhibit the synthesis of the prostaglandins that are required to protect the gastrointestinal mucosa, resulting, ultimately, in the formation of ulcers in the gastrointestinal tracts of patients who are chronic users of these compounds. A compound that inhibited COX-2 without affecting COX-1 would be expected to be anti-inflammatory and lack the gastrointestinal side effects that are common to compounds that inhibit both isoforms of cyclooxygenase [3].

Rofecoxib [compound **I**, 4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one, Fig. 1] has

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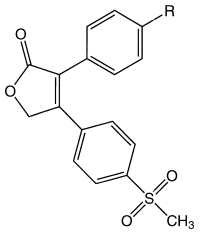

Compound I: R = H
Compound II: R = CH_3

Fig. 1. Structures of compound **I** and the internal standard, **II**.

been found to be a COX-2 specific inhibitor at the recommended clinical doses for osteoarthritis and pain relief. A method for the determination of **I** in plasma obtained following the oral administration of the compound was required in order to support human pharmacokinetic studies of this compound. A high-performance liquid chromatographic assay for the determination of **I** in human plasma utilizing liquid–liquid extraction for sample preparation followed by high-performance liquid chromatography (HPLC) with post-column photochemical derivatization and fluorescence detection is the subject of this publication.

The use of HPLC with post-column photochemical derivatization–fluorescence detection has been previously reported for the determination of various drugs in biological fluids [4–8]. In many cases the technique has been reported to be highly competitive with HPLC with tandem mass spectrometric detection in terms of speed of analysis, selectivity and sensitivity [9]. Although the technique is not universal in nature, in selected cases, where the unique chemical structure of the analyte makes it amenable to an efficient photochemical rearrangement or decomposition that leads to the formation of highly fluorescent products, the method offers exceptional selectivity and sensitivity. The example presented here illustrates the application of post-column derivatization methodology for the sensitive determination of **I** in plasma samples originating from studies conducted during the clinical development program of rofecoxib.

2. Experimental

2.1. Materials

Compound **I** was obtained from the Chemical Data Department of Merck Research Labs. (Rahway, NJ, USA). Compound **II** (internal standard, Fig. 1) was synthesized by S. Leger of the Medicinal Chemistry Department of Merck Frosst Canada (Kirkland, Quebec, Canada). The standard of 6-(methylsulfonyl)phenanthro[9,10-C]furan-1(3H)-one (compound **III**, Fig. 3) was provided by Dr. R. Tillyer of the Department of Process Research of Merck Research Labs. Solvents (Omnisolve, HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received.

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump, a Waters (Milford, MA, USA) WISP 715 automatic injector, an Astec (Whippany, NJ, USA) Beam Boost photochemical reactor equipped with a 254 nm lamp and a 10 m reaction coil (0.3 mm I.D.), and a Perkin-Elmer LC240 fluorescence detector. The analog output from the detector was connected to a PE-Nelson (San Jose, CA, USA) Access-Chrom data acquisition system via a PE-Nelson Model 941 interface.

Absorption and fluorescence spectra were obtained

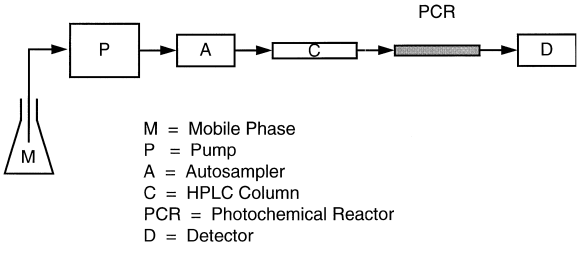

Fig. 2. Block diagram of the HPLC system used for the analysis of **I** in human plasma.

using a diode array spectrophotometer (HP 8452, Hewlett-Packard, Palo Alto, CA, USA) and an Hitachi (Danbury, CT, USA) Model F-4500 spectrofluorometer.

An API III+ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer atmospheric pressure chemical ionization interface was used to obtain mass spectra of the photolysis products of **I** and **II**. The mass spectrometer was operated in the negative-ion mode at unit mass resolution. The nebulizer pressure was set at 80 p.s.i. (1 p.s.i.=6894.76 Pa). The interface discharge needle was maintained at a current of 4 μA and the sampling orifice was set at −40 V. Product ion spectra were obtained at a collision energy of −15 V.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–water (35:65, v/v). The mobile phase was filtered through a 0.20-μm nylon membrane filter prior to use. A BDS-Hypersil C_{18} analytical column (100×4.6 mm, 5 μm packing) (Keystone Scientific, State College, PA, USA) preceded by a threaded guard cartridge column (20×4 mm) packed with the same material was used for the separation. The mobile phase was delivered through the columns at a flow rate of 1.2 ml/min. The columns were operated at ambient temperature (approximately 22°C).

The sample injection volume was 150 μl. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 375 nm.

2.4. Preparation of standards

A 10 μg/ml solution of **I** was prepared by weighing 1.0 mg of the compound into a 100-ml volumetric flask and dissolving the material in about 50 ml of acetonitrile before filling the flask to volume with acetonitrile. A 1.0 μg/ml stock solution was prepared by diluting 5 ml of the 10 μg/ml to 50 ml with acetonitrile, while a 0.2 μg/ml stock solution was prepared by diluting 10 ml of the 1.0 μg/ml stock solution to 50 ml with acetonitrile.

Working standards of 2 and 1 μg/ml were prepared by dilution of the 10 μg/ml stock solution with acetonitrile. Working standards of 0.4, 0.2, and 0.1 μg/ml were prepared by dilution of the 1.0 μg/ml stock solution and working standards of 0.04, 0.02, and 0.01 μg/ml were prepared by dilution of the 0.2 μg/ml stock solution with acetonitrile. All standard solutions were found to be stable for at least two weeks when stored protected from light at room temperature.

Plasma standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma contained within a 15-ml disposable glass centrifuge tube. The resulting standards were used to quantitate samples containing **I** at concentrations between 0.5 and 100 ng/ml. Samples containing higher concentrations of **I** were diluted with control plasma prior to analysis.

2.5. Plasma extraction procedure

Frozen plasma samples were thawed at room temperature and vigorously vortexed for approximately 1 min. A 1-ml aliquot of the well mixed plasma sample was pipetted into a 15-ml disposable glass centrifuge tube. A 50-μl aliquot of acetonitrile was then added to the sample to make the volume of the samples identical to that of the plasma standards. A 25-μl volume of a 0.4 μg/ml solution of internal standard in acetonitrile (compound **II**, Fig. 1) was then added to the tubes containing the plasma standards and the samples. The contents of the tubes were mixed by vortex. A 1-ml volume of 0.1 *M* acetate buffer (pH 5) was added to each tube, the contents were vortexed, and 8 ml of extraction solvent (methylene chloride–hexane, 50:50, v/v) was added. The tubes were sealed with PTFE lined screw caps (Qorpak 5201, Fisher Scientific), and the contents of the tubes were mixed for 5 min on a flat bed shaker. Following mixing, the tubes were centrifuged for 5 min at 1500 *g* to affect a phase separation of the contents. The tubes were placed in a dry ice–acetone bath, and after the lower aqueous layers were frozen, the entire upper organic layers were decanted into 100×16 mm disposable glass culture tubes. The tubes containing the organic layers were placed in a Turbo-Vap evaporator (Zymark, Hopkin-

ton, MA, USA) set at 50°C, and the solvent was evaporated under a stream of nitrogen. The resulting residue in each tube was reconstituted in 1 ml of mobile phase and a 200-μl aliquot of the reconstituted extract was transferred to a polymethylpentene plastic limited volume insert contained in an autosampler vial. The vials were capped and placed in a autosampler tray to await injection.

2.6. Fluorescence quantum efficiency

The absolute fluorescence quantum efficiency (Φ_f) for **III** was determined relative to phenanthrene as standard (Φ_f=0.13 in ethanol [10]). Corrected fluorescence spectra of **III** and phenanthrene were utilized for calculations of relative fluorescence intensities in order to correct for differences in the emission spectra of the compounds. The measurement of Φ_f was performed using a similar procedure as described previously [11].

3. Results and discussion

3.1. Spectroscopic and photochemical properties of rofecoxib

In order to establish the best conditions for the sensitive detection of **I**, a series of spectroscopic and photochemical studies were performed. The ultraviolet (UV) spectrum of the compound, obtained in mixtures of acetonitrile–buffer (50:50, v/v), indicated the presence of two absorption bands with maxima at <210 nm and 272 nm. The positions of the maxima of these UV bands were found to be relatively independent of pH. The molar extinction coefficient ($\in$) of the peak at 272 nm ranged from 13 500 M^{-1} cm^{-1} at pH 3.0 to 15 000 M^{-1} cm^{-1} at pH 11.8. **I** was found to be practically non-fluorescent at acidic, neutral or basic pH.

Exposure of solutions of the compound to UV light (254 nm) led to significant changes in its UV spectrum. New absorption bands with maxima at 220, 250, and 320 nm appeared. Additionally, the solutions, after photolysis, exhibited significant fluorescence. The fluorescence emission maximum varied from 410 nm at pH 11.8 to 431 nm at pH 3.0. The kinetics of the photochemical reaction appeared to be rapid, as the UV and fluorescence spectra of solutions exposed to UV light for 2 min were identical, in terms of the wavelength of the absorption maximum and fluorescence emission intensity, to those of solutions of the identical concentration exposed under the same irradiation conditions for only 30 s. The UV and fluorescence spectra of solutions of **I** exposed to UV light closely resembled those of solutions of 6-(methylsulfonyl)phenanthro[9,10-C]furan-1(3H)-one (compound **III**), indicating that stilbene–phenanthrene-like cyclization [12–14] was probably responsible for the photochemical changes associated with **I** (Fig. 3).

To further study the photochemistry of **I**, a photochemical reactor consisting of a 5 m PTFE reaction coil capable of withstanding high (>1000 p.s.i.) pressure and a 254 nm lamp was positioned in an HPLC system between the injector and an HPLC column (BDS-Hypersil C_{18}, 250×4.6 mm). Using a mobile phase of acetonitrile–water (35:65, v/v) at a flow rate of 1.2 ml/min, the retention times of compounds **I** and **III**, injected into the system with the 254 nm lamp off, were found to be approximately 12 and 16 min, respectively. UV absorbance at 272 nm was used for detection. Injection of 100 μl of a 10 ng/ml solution of **I** into the system with the 254 nm lamp on resulted in the almost total (>95%) disappearance of the peak at 12 min and the appearance of a peak at 16 min, the retention time of **III**, indicating that irradiation of **I** in a mobile phase of acetonitrile–water (35:65, v/v) resulted in the formation of **III**. Furthermore, based on these results, it can be concluded that the photochemical reaction was more than 95% complete within the residence time of the reactor (approx. 18 s).

Additional confirmation regarding the structure of

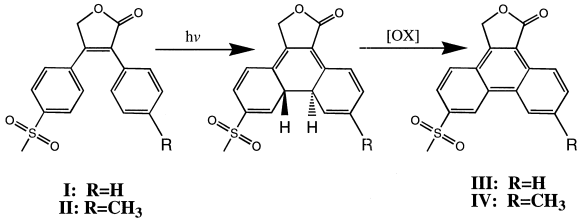

Fig. 3. Photocyclization of **I** and **II**.

the photolysis product of **I** was obtained by connecting the HPLC system with the high pressure reaction coil to a tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) HPLC interface. Mass spectra, acquired in negative ionization mode, of the chromatographic peaks corresponding to **III** and the photolysis product both exhibited the same deprotonated molecular ion at m/z 311. Furthermore, the product ion spectra of these compounds, obtained by subjecting the m/z 311 ions to collision induced fragmentation, were practically the same for **III** and the product of photolysis; major product ions at m/z 283 and m/z 255 were observed in both cases. These data clearly confirm that **I** undergoes stilbene–phenanthrene-like photocyclization and that the nature of product of this reaction is as depicted in Fig. 3.

The mass spectrum, acquired in negative ionization mode, of the chromatographic peak corresponding to the photolysis product of the internal standard (**II**) exhibited a deprotonated molecular ion at m/z 325. Major products ions at m/z 297 and 269 were observed when the m/z 325 ion was subjected to collision induced fragmentation. These data indicate that **I** and **II** undergo similar photochemistry (Fig. 3).

Following these experiments, the photochemical reactor was re-positioned between the column outlet and the inlet of a fluorescence detector. With the photochemical reactor lamp on, approximately 70 pg of **I** injected on column could be detected with a signal-to-noise ratio of approximately 5:1. The low limit of detection can be attributed to the relatively high fluorescence quantum efficiency (Φ_f) of **III**, which was determined to be 0.57 in ethanol. Based on these results, the post-column photochemical derivatization/fluorescence detection system shown in Fig. 2 was used to detect the analytes.

3.2. Extraction procedure development

Initial experiments to develop a suitable sample preparation procedure focused on the use of liquid–liquid extraction. Compound **I** was found to extract quantitatively from aqueous buffers over the pH range of 1 to 9 using either methyl-*tert*.-butyl ether (MTBE) or a mixture of hexane–methylene chloride (50:50, v/v) as the extraction solvent. MTBE extracts of spiked plasma samples that were buffered between pH 1 and 9 were found to contain endogenous peaks that interfered with the detection of the analytes. Plasma samples that were buffered to pH 5 and extracted with hexane–methylene chloride (50:50, v/v) were found to be free of interferences. Hence, this procedure was used for the assay.

3.3. Extraction recovery

The recovery of the extraction method was determined by comparing the responses of neat standards containing **I** with those of extracted plasma standards. The results (Table 1) indicate that the mean recovery for **I** over the concentration range of the assay was 96.8%. The recovery of the internal standard was found to be comparable to that of **I**.

3.4. Assay selectivity

Fig. 4 shows chromatograms of extracted drug-free plasma (A), a plasma standard containing 1 ng/ml of **I** and 10 ng/ml of **II** (B) and a plasma sample taken from a subject after receiving a 50 mg

Table 1
Recovery of **I** from human plasma

Concentration (ng/ml)	Mean (n=5) recovery of **I** (%)	RSD (%)
0.5	96.8	4.3
1.0	98.2	4.2
2.0	97.8	1.5
5.0	94.7	0.4
10.0	95.6	1.4
20.0	98.2	1.4
50.0	96.2	0.8
100.0	97.1	0.1

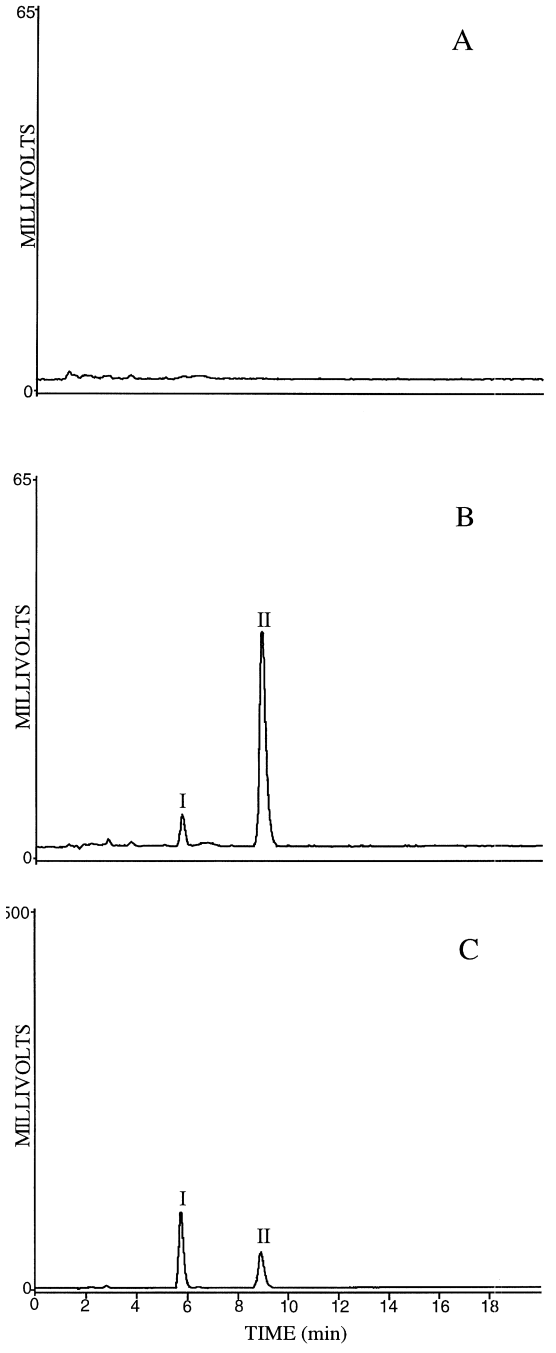

Fig. 4. Representative chromatograms of (A) control human plasma, (B) plasma spiked with 1 ng/ml **I** and internal standard (10 ng/ml), (C) plasma sample from human subject 3 hours after administration of 50 mg **I**. Internal standard, **II** added at 10 ng/ml following a 1:10 dilution of the sample. The concentration of **I** is equivalent to 159.2 ng/ml.

dose of **I** (C). A comparison of Fig. 4A with 4B illustrates that no endogenous peaks elute at the retention times of **I** or **II**. The selectivity of the method is further illustrated by the fact that all pre-dose plasma samples from subjects involved in clinical trials were free of interfering peaks.

3.5. Linearity

Weighted (weighting factor=$1/y$, where y=peak height ratio of **I**/**II**) least-squares regression calibration curves, constructed by plotting the peak height ratio of **I** to **II** versus the standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration range 0.5–100 ng/ml **I**. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentration and the experimentally determined standard concentration calculated from the regression equation.

3.6. Assay precision and accuracy

Replicate (n=5) standards containing **I** were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentration as well as the relative standard deviation (RSD) of the plasma replicate standards are shown in Table 2.

Quality control (QC) samples containing **I** at concentrations of 1.5 ng/ml (low QC) and 75 ng/ml (high QC) were prepared and frozen (−20°C) in

Table 2
Within-day precision and accuracy data for the assay of **I** in plasma

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b] (%)
Nominal	Mean (n=5) analyzed		
0.5	0.49	6.9	98.0
1	1.00	1.7	100.0
2	2.05	1.0	102.5
5	4.99	0.8	99.8
10	10.52	1.1	100.5
20	19.83	0.8	99.2
50	50.95	1.3	101.9
100	99.19	0.8	99.2

[a] Expressed as the relative standard deviation (RSD, %).
[b] Expressed as [(mean observed concentration)/(nominal concentration)×100].

Table 3
Inter-day variability of the assay of **I** in plasma as assessed by RSDs of low and high quality control samples

Nominal concentration (ng/ml)	Mean analyzed concentration[a]	RSD (%)
1.5	1.48	4.62
75	73.95	1.16

[a] Results represent 24 standard curves over a one-month period.

1.25-ml aliquots. Two pairs of quality control samples were analyzed with each of 24 standard curves over a one-month period. The results (Table 3) indicate that the between-day variability (RSD) of the method is under 5%. The results also indicate that frozen plasma samples containing **I** appear stable for at least one month.

3.7. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded a within-day RSD of less than 10% and a within-day accuracy between 90 and 110% of nominal concentration, was 0.5 ng/ml **I**.

3.8. Clinical sample analysis

The liquid–liquid extraction–post-column photochemical derivatization–fluorescence assay for the determination of **I** in human plasma has been used to support several clinical studies. Representative plasma levels from subjects receiving a 10 mg oral dose of **I** are shown in Table 4.

4. Conclusion

A HPLC assay using post-column photochemical derivatization has been developed for the determination of **I** in human plasma. The method has been found to be precise, accurate and suitable for the analysis of plasma samples collected during human clinical studies of **I**.

Table 4
Plasma concentrations (ng/ml) of **I** following single-dose administration of 10 mg to selected healthy volunteers

Subject	Concentration (ng/ml)							
	Time (h)							
	0	1	3	5	12	20	24	48
1	nd[a]	30.4	45.5	96.5	44.0	32.9	26.0	2.4
2	nd	60.5	70.6	65.1	37.0	23.9	17.5	2.3
3	nd	63.0	71.1	81.7	47.3	32.4	24.2	4.1

[a] nd=Not detected.

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