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## Determination of Rofecoxib in Serum with Pre-column Derivatization and Fluorescence Detection

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**Abstract:** A HPLC method for determination of rofecoxib in human serum was presented. The method is based on pre-column derivatization of analyte to a phenanthrene derivative of drug. Rofecoxib and internal standard were extracted from serum using liquid-liquid extraction. Upon exposure to UV light, drug was found to undergo a photocyclization reaction giving a species with high absorbance. Validation of the method has been studied in the concentration range of 2-100 ng mL<sup>-1</sup>.

**Key words:** Rofecoxib, HPLC, Serum, derivatization

### INTRODUCTION

Rofecoxib is a cox-2 selective inhibitor with anti-inflammatory property and lack the gastrointestinal toxicity<sup>[1]</sup>. Several methods have been published for quantitation of rofecoxib (ROF) in biological fluids. Chavez-Eng *et al.*<sup>[2]</sup> introduced a method for the determination of ROF in human plasma by HPLC and atmospheric pressure chemical ionization tandem mass spectrometric detection in negative ionization mode. Subsequently they developed their method to support the clinical oral bioavailability study of ROF<sup>[3]</sup>. A HPLC method for the determination of ROF in plasma using a fluorescence detector and post-column UV photocyclization of the parent drug has been reported<sup>[4]</sup>. An isocratic reversed phase-liquid chromatographic method has been developed for bulk and pharmaceutical dosage form in which a degraded product was formed upon exposure of ROF to UV light<sup>[5]</sup>. This cyclized product (Fig. 1) was observed as a potential impurity of the rofecoxib photodegradate<sup>[6]</sup>. This phenanthrene analog of ROF was formed in the post-column derivatization method reported by Woolf<sup>[4]</sup>. With inspiration of Wolf method and Radhakrish report<sup>[5]</sup>, we designed an easy HPLC pre-column derivatization of ROF in serum with UV detection in the present research.

### MATERIALS AND METHODS

HPLC grade acetonitrile and dichloromethane were analytical grade that were purchased from Merck

Company (Darmstadt, Germany). HPLC grade water obtained by double distillation in glass and purification through a Mill-Q waters purification system (Millipore, Bedford, MA). Rofecoxib was extracted from vioxx tablets (25 mg), (Merck, Kirkland, Quebec, Canada, lot Hoos 440) and their purity was checked according to the literature procedure<sup>[7]</sup>.

**Chromatographic condition and instrumentations:** The waters HPLC system (waters Assoc. Milford, MA) employed consisted of a 510 pump, a Rheodyne injector equipped with a 20 µL sample loop and a waters 474 fluorescence detector adjusted at 270 nm (excitation) and 350 nm (emission) connected to a 746 data module integrator. The mobile phase was consisted of a mixture of acetonitrile-water (40:60). The mobile phase was prepared daily and delivered at a flow rate of 1 mL min<sup>-1</sup>. Separation was achieved using a C18 analytical column (150×4.6 mm, i.d., 5 µ, Tracer). All chromatographic separations were performed at room temperature.

The <sup>1</sup>H NMR spectra were recorded on a Varian unity plus spectrometer at 400 MHz. Chemical shifts (δ) are given in part per million (ppm) relative to TMS. Coupling constants (J) are given in hertz (Hz).

**Sample preparation:** A stock solution of ROF and diazepam, IS, (1 mg mL<sup>-1</sup>) was prepared in acetonitrile and kept in a light proof container and refrigerated in order to reduce its degradation and photocyclization. Under these circumstances, the solution was stable at least for two months. Working standard solutions were prepared daily by diluting the stock solution with acetonitrile.

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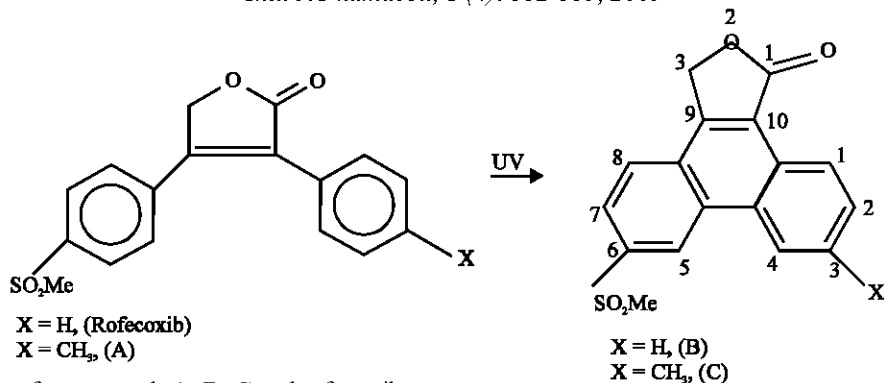


Fig. 1: Structures of compounds A, B, C and rofecoxib

Frozen human serum samples were obtained from Blood transfusion center (Red Crescent organization), thawed and allowed to reach room temperature. A 1 mL aliquot of serum was placed into a 10 mL glass screw-capped tube, 20  $\mu$ L of IS solution (1  $\mu$ g mL<sup>-1</sup>) and 20  $\mu$ L working standard solution of ROF were added and mixed. Five milliliter of dichloromethane was added to each test tube and vortex mixed for 1 min at 500 rpm, then centrifuged for 10 min at 3500 rpm.

Upper layer was discarded and the organic phase layer was transferred to a clean test tube. Dichloromethane was dried by adding about 50 mg anhydrous sodium sulfate, transferred to a next test tube and evaporated to dryness under N<sub>2</sub> stream. The residue was reconstituted in 200  $\mu$ L acetonitrile and transferred to a 3 mL quartz cell and exposed to UV beam (254 nm) for 10 min at a distance of 10 cm from source of UV. Twenty five microliter of solution was injected to HPLC under described procedure.

**Calibration curve and recovery:** Working standard solutions were added to thawed serum to yield concentrations ranging from 2-100 ng mL<sup>-1</sup>. Calibration curve from serum were obtained by plotting peak area ratio of ROF to IS against ROF concentrations. The recovery was calculated by comparing peak area ratio obtained from serum samples with area ratio resulted after injecting standard solutions at the same theoretical concentration.

Synthesis of 6-(methylsulfonyl)phenanthro [9,10,C] furan-2-(5H) one (compound B, Fig. 1) and 3-methyl-6-(methylsulfonyl)phenanthro [9,10,C] furan-2-(5H) one (compound C, internal standard).

Twenty milligram of ROF (or compound A) was dissolved in 500 mL acetonitrile and stirred under UV radiation for 12 h at room temperature. The solvent was evaporated under vacuum and the residue was purified by TLC (20 $\times$ 20 plates) with a mobile phase consisted of

chloroform: ethyl acetate (1:1). The fast moving spot (comparing to the starting material) was crystallized from methanol to give compound B and C. The structure of compound B and C was confirmed by <sup>1</sup>H NMR.

**Compound B:** <sup>1</sup>HNMR (CDCl<sub>3</sub>)  $\delta$  9.40 (d, J=1.6 Hz, H<sub>5</sub>), 9.08 (m, 2H, H<sub>1</sub> and H<sub>4</sub>), 8.34 (d, J=8.3 Hz, 1H, H<sub>8</sub>), 8.28 (dd, J=8.3 Hz and J=1.6 Hz, 1H, H<sub>7</sub>), 7.90 (m, 2H, H<sub>2</sub> and H<sub>3</sub>), 5.85 (s, 2H, CH<sub>2</sub>, furanone), 3.45 (s, 3H, SO<sub>2</sub>CH<sub>3</sub>).

**Compound C:** <sup>1</sup>HNMR (CDCl<sub>3</sub>)  $\delta$  9.39 (s, 1H, H<sub>5</sub>), 9.05 (d, J=8.8 Hz, 1H, H<sub>7</sub>), 8.61 (s, 1H, H<sub>4</sub>), 8.21 (d, J=8.8 Hz, 1H, H<sub>8</sub>), 8.04 (d, J=8.2 Hz, 1H, H<sub>2</sub>), 7.70 (d, J=8.2 Hz, 1H, H<sub>1</sub>), 7.79 (d, J= 7.5 Hz, 1H, H<sub>1</sub>), 5.85 (s, 2H, CH<sub>2</sub>, furanone), 3.23 (s, 3H, SO<sub>2</sub>CH<sub>3</sub>), 2.63(s, 3H CH<sub>3</sub>).

Synthesis of 4-(4-methylsulfonyl)-3-(4-methylphenyl)-5H-furane-2-one (compound A, Fig. 1).

For synthesis of compound A, 4-methyl phenyl acetic acid was used instead of phenyl acetic acid similar to the literature procedure to prepare of rofecoxib<sup>[8]</sup>. <sup>1</sup>HNMR (CDCl<sub>3</sub>)  $\delta$  7.92 (d, J=8.4 Hz, 2H), 7.52 (d, J=8.4 Hz, 2H), 7.28 (d, J=7.6 Hz, 2H), 7.19 (d, J=7.6 Hz, 2H), 5.15 (s, 2H, CH<sub>2</sub>, furan), 3.04 (s, 3H, SO<sub>2</sub>CH<sub>3</sub>), 2.38(s, 3H, CH<sub>3</sub>).

## RESULTS

Under the chromatographic conditions described, phenanthrene derivative of ROF and IS were well resolved in serum. They don not contain any interfering peak showing a retention time similar to that of ROF-derivative which was found to have an retention time of 4.45 min using a mobile phase of acetonitrile-water (40:60) at a flow rate of 1 mL min<sup>-1</sup> (Fig. 2). It was shown that photochemical reaction for conversion of ROF to compound B was more than 95%<sup>[4]</sup> that was similar with our findings. The structure of peak with retention time of 4.45 was confirmed by the comparison of the retention

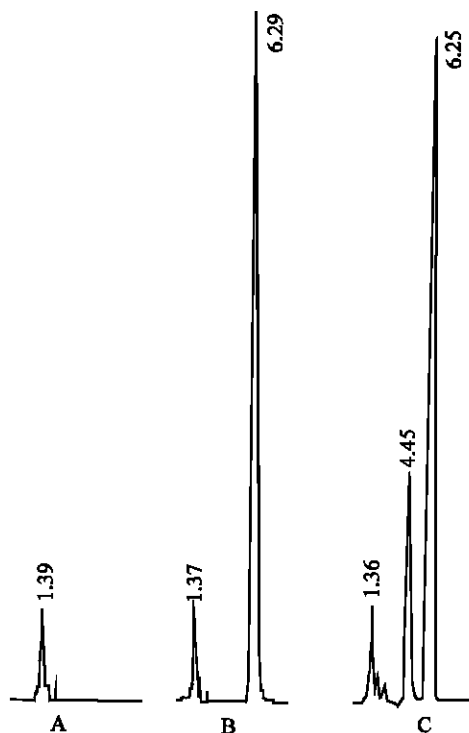


Fig. 2: HPLC chromatograms of ROF and IS, A) Drug free serum B) Serum spiked with IS (20 ng mL<sup>-1</sup>) and C) Serum spiked with 10 ng mL<sup>-1</sup> of ROF (4.45) and IS (6.25)

time of the compound B in spiked serum sample and synthesized product under the same chromatographic condition.

The calibration curve for determination of ROF in serum was linear over range of 2-100 ng mL<sup>-1</sup> (2, 5, 10, 20, 50, 100) and corresponding regression equation was  $Y = 0.0391 X + 0.1898$  ( $r^2 = 0.9993$ ), where Y is the peak area ratio of compound B to IS and X is the ROF concentration. The CV values for all six selected concentrations were less than 10.52. The reproducibility of the method was determined by repeating three replicate serum samples on each three-separated day. According to the intra-day and inter-day data good precision and accuracy were observed on the entire concentration range. The results are presented in Table 1. The Limit of Quantification (LOQ) of the method defined as the minimum concentration that could be measured with CV<10% was found to be 2 ng mL<sup>-1</sup>. The Limit of Detection (LOD) with a S/N ratio 5:1 was 0.1 ng mL<sup>-1</sup> in serum. The recovery of drug was determined by comparing the peak area ratio for compound B from Spiked serum and a standard solution of synthesized B with the same concentration. Three-concentration range, i.e. 10, 20,100 ng mL<sup>-1</sup> were investigated for recovery studies. The mean of recovery from the serum was 93.16% (Table 2).

Table 1: Accuracy and precision in spiked plasma (n=9, 3 sets for three days)

Concentration added (ng mL <sup>-1</sup> )	Concentration found (mean±SD) ng mL <sup>-1</sup>	CV (%)	Accuracy (%)
Intra-day (n=3)			
2	1.94±0.08	4.09	97.19
10	10.87±0.74	6.78	108.70
50	50.41±0.42	0.83	100.82
100	105.05±10.45	9.95	105.05
Inter-day (n=3)			
2	1.60±0.38	23.94	80.05
10	8.83±0.20	2.29	88.29
50	47.05±2.36	5.02	94.11
100	96.25±5.43	5.65	96.25

Table 2: Extraction efficacy of ROF from serum at various concentrations, mean±SD (n=3)

Concentration (ng mL <sup>-1</sup> )	Percentage of extraction±SD
10	94.61±3.79
20	90.16±3.45
100	94.71±7.65

Mean of recovery is 93.16%

## DISCUSSION

The cyclic product (compound B) was reported as only photo-degraded product when the solution of ROF was exposed under UV light<sup>[4-6]</sup>. Photo cyclization of ROF led to significant changes in its UV spectrum property<sup>[4]</sup>. Several new absorption bands with remarkable intensity comparing to rofecoxib were observed at 230- 260 nm<sup>[5]</sup>.

Furthermore, based on the previous study solution of ROF, after photolysis, exhibited significant fluorescence property. The use of HPLC with post-column photochemical derivatization–fluorescence detection has been reported for the determination of various drugs in biological fluids. In many cases the technique has been reported to be highly competitive with HPLC-tandem mass spectrometric detection in terms of speed of analysis, selectivity and sensitivity but unfortunately, the technique is not universal in nature<sup>[4]</sup>. The assessment of ROF according to Wolf method requires a post-column UV reactor for photocyclization before detection with fluorescence detector. A long retention time for ROF and IS, 13 and 24 min, respectively, was reported therefore, at least 30 min run time for one sample is required<sup>[7]</sup>. We developed a new assay method for determination of rofecoxibe in serum with a short run time that is based on simply derivatization and adequate sensitivity for routine assessment and pharmacokinetic studies.

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**REFERENCES**

1. Sorbera, L.A., P.A. Leeson and J. Castaner, 1998. Rofecoxib, anti-inflammatory cyclooxygenase-2 inhibitor. *Drug Future*, 23: 1287.
2. Chavez-Eng, C.M., M.L. Contanzer and B.K. Matuzewski, 2000. Determination of rofecoxib (MK-0966), a cyclooxygenase-2 inhibitor, in human plasma by high-performance liquid chromatography with tandem mass spectrometric detection. *J. Chromatogr., B* 748: 31-39.
3. Chavez-Eng, C.M., M.L. Contanzer and B.K. Matuzewski, 2002. High-performance liquid chromatographic-tandem mass spectrometric evaluation and determination of stable isotope labeled analogs of rofecoxib in human plasma samples from oral bioavailability studies. *J. Chromatogr., B* 767: 117-129.
4. Woolf, E. and B. Matuszewski, 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.
5. Radhakrishna, T., D. Sreenivas Rao and G. Reddy, 2001. LC determination of rofecoxib in bulk and pharmaceutical formulations. *J. Pharm. Biomed. Anal.*, 26: 617-628.
6. Mao, B., A. Abraham, Z. Ellison, R. Hartman, S.V. Proabhu, R.A. Reamer and J. Wyvratt, 2002. Examination of rofecoxib solution decomposition under alkaline and photolytic conditions. *J. Pharm. Biomed. Anal.*, 28: 1101-1113.
7. Sattari, S. and F. Jamali, 2000. High performance liquid chromatographic determination of cyclooxygenase inhibitor rofecoxib in rat and human plasma. *J. Pharm. Pharmaceut. Sci.*, 3: 312-316.