# An Improved HPLC method of Analysis of Rofecoxib

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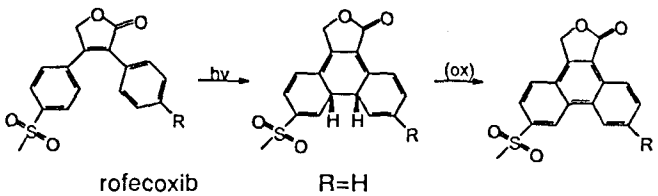
**An improved, simple, specific and more sensitive method for quantitative determination of rofecoxib from the human plasma is described. The method consists of pre-column photoactivation of rofecoxib in acetonitrile by exposure to UV light at 366 nm. The drug probably undergoes a stilbene-phenanthrene like photocyclization reaction with resulting formation of a highly fluorescent species. Subsequent injection on C18e column in HPLC with fluorescent detector at 260 nm excitation and emission at 381 nm with a mobile phase consisting of water:acetonitrile (55:45 v/v) could determine rofecoxib quantitatively in the concentration range of 10 to 350 ng/ml using 20 µl of samples. The assay has been validated and has been successfully utilized in clinical studies.**

Rofecoxib, a selective COX-2 inhibitor used in osteoarthritis and management of acute pain probably undergoes stilbene-phenanthrene like photocyclization reaction when exposed to UV light yielding highly fluorescent species[1], which is shown in the fig 1. The use of pre-column and post column derivatization in chromatography are mainly to improve the detection level, selectivity and sensitivity of the method[2]. This is particularly true in case of fluorescence detection, utilized for determination of trace levels of bioactive compounds in complicated matrices such as biological and environmental samples. The groups considered include amines and amino acids[3], carboxylic acids[4-6], alcohols[7], phenols,[8-10] thiols[11], estrogents[12] and aldehydes and ketones[13]. In addition, photolytic derivatization for aspartame[14] and bentazone[15] have also been reported.

HPLC methods with post-column photochemical derivatization and fluorescence detection for the determination of rofecoxib in biological fluids have been reported[1]. The method is quite complex and requires a 10-m reaction coil equipped with a photochemical reactor, which is tedious, expensive and not sufficiently sensitive. The kinetics of the photochemical reaction include exposure of the solution of rofecoxib to UV light, which in turn leads to significant changes in its fluorescent spectrum due to photolysis. This type of reaction has been followed earlier for determination of methotrexate[16], sulindac[17], fenbufen[18], and diclofenac[19] in biological fluids via post-column photochemical derivatization by using fluorescence detection. This reaction greatly improves the sensitivity and selectivity of the method.

The work reported here, uses the principle of pre-column photoactivation in place of cumbersome post-column photoactivation and HPLC with fluorescence detector instead of LC-MS.

The working standard of rofecoxib (purity 99.86%) was obtained from Micro Labs Ltd., Hosur and HPLC grade solvents including methanol, acetonitrile, n-hexane, dichloromethane (DCM) were obtained from E-Merck, Mumbai. Drug-free human plasma was obtained from Blood Bank of KEM Hospital, Mumbai. All the other reagents were of high purity and were used as received.




rofecoxib R=H

**Fig. 1: Photocyclization reaction of rofecoxib[1].**

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HPLC system consisting of an isocratic pump (Jasco PU980), an automatic injector (Jasco 851-As) and a fluorescence detector (Jasco FP-750), was used along with a UV/Vis spectrophotometer (Jasco V-550) and a spectrofluorimeter (Jasco FP-750). The photochemical reactor was equipped with 366 nm lamp with bottom and side ways reflectors enclosed in a dark area of 22x22".

Standard 100-µg/ml solution of rofecoxib was prepared in acetonitrile. It was further diluted as and when required in acetonitrile. The working standards with concentration ranging from 45 to 2000 ng/ml of the drug were prepared in mobile phase [water:acetonitrile (55:45 v/v)] from stock solution with suitable dilutions. All the standard solutions were stored in a refrigerator and were used within two weeks from the date of preparation.

Quality control samples were prepared by spiking rofecoxib into drug-free plasma with different quantities of stock solution to get final concentrations of rofecoxib, ranging from 10 to 350 ng/ml. These samples were stored at – 20° until analyzed. The quality control sample was allowed to thaw. One milliliter of this sample was withdrawn accurately, added into a disposable glass centrifuge tube and 1 ml of acetate buffer (pH 7.0, 500 mM) was added. The mixture was vortexed for 2 min. The drug was extracted with 8 ml of extraction solvent [dichloromethane (DCM):n-hexane], the contents were mixed for 40 min on a rotating shaker and the tubes were centrifuged for 15 min at 1800 rpm. The upper organic layer was transferred to clean test tubes and evaporated using Zymark Turbovap LV evaporator at 50° under a stream of nitrogen. Finally, tubes containing residue were activated in specially designed photoactivation chamber for 20-25 min. The resulting residue was reconstituted with 200 µl of mobile phase and 20 l was injected on a Lichrocart RP-18e analytical column (5 µm) preceded by guard column packed with same material, which was used for separation. The mobile phase containing a mixture of water:acetonitrile (55:45 v/v) filtered through 0.20 µm nylon membrane filter, degassed and delivered through the column at a flow rate of 1 ml/min. The column was operated at ambient temperature (24 ±1°). The fluorescent detector was set at λ excitation of 260 nm and λ emission of 381 nm. Retention time of rofecoxib was observed between 7.5 to 8 min.

The pre-photoactivation UV scan of rofecoxib in methanol showed peaks at 225.5 nm (Abs 3.319), 284 nm (Abs 2.853) and 287.5 nm (Abs 2.850), post-photoactivation with UV at 366 nm showed new peaks appearing at 242.5 nm

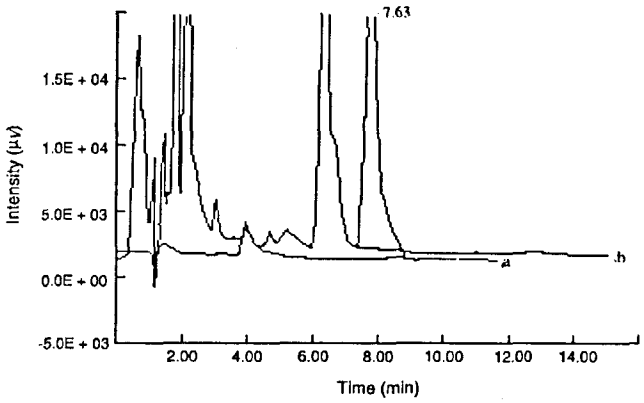


**Fig. 2: a. Chromatogram of extracted drug-free plasma. b. Chromatogram of extract of rofecoxib, spiked containing 50 ng/ml.**

(Abs- 3.089) and 259.5 nm (Abs 3.307) in addition to a shift in the peak from 225.5 nm (Abs- 3.319) to 229 nm (with Abs 3.580). The cyclization was also associated with the dissociation of the peaks at 288 nm and 287.5 nm. These observations are different from those made by Woolf *et al.*[1]

Photoactivation at 366 nm increased the sensitivity of the method as is evident from the values of LOD, LOQ and Linearity Range after photoactivation as compared to these values obtained without photoactivation. The values of LOD, LOQ and Linearity Range in ng/ml obtained were 3, 10 and 10-350 after photoactivation as compared to 16, 50 and 50-250 without photoactivation respectively. The above-validated results are recorded in Table 1.

The spectrofluorimetric scan of rofecoxib (100 ng/ml) prior to photoactivation showed excitation at 320 nm and emission at 382 nm which is changed to excitation at 260

TABLE 1: LIMIT OF DETECTION, LIMIT OF QUANTITATION AND LINEARITY RANGE OF ROFECOXIB

| Parameters | No photoactivation | After activation |
|---|---|---|
| LOD (ng/ml) | 16 | 3 |
| LOQ (ng/ml) | 50 | 10 |
| Linearity range (ng/ml) | 50-250 | 10-350 |

n represents replicate number of injections, LOD stands for Limit of Detection and LOQ stands for Limit of Quantification.

TABLE 2: ASSAY PRECISION OF ROFECOXIB

| Amount added | Intra day (ng/ml) | | | Inter day (ng/ml) | | |
|---|---|---|---|---|---|---|
| (ng/ml) | 25 | 150 | 300 | 25 | 150 | 300 |
| Amount found (ng/ml) | 25.05 | 149.06 | 300.69 | 24.87 | 149.26 | 301.99 |
| SD (±) | 0.49 | 1.41 | 2.43 | 0.53 | 2.01 | 3.35 |
| % CV | 1.99 | 0.95 | 0.81 | 2.15 | 1.35 | 1.11 |

n represents replicate number of injections. SD represents standard deviation and % CV represents percent coefficient of variation.

nm and emission at 381 nm after photoactivation. This was due to production of flurophore and enabled the method to be used selectively and specifically for the determination of rofecoxib with higher sensitivity as stated above. Recovery was determined by comparing the responses of calibrators (standards) containing rofecoxib with those of extracted plasma (quality control) samples. The results indicated that the mean recovery for rofecoxib over the concentration range of assay was 98.4% with %CV not more than 3. Assay selectivity was carried out by injecting drug-free plasma and plasma containing 50 ng/ml of rofecoxib. Comparison of the profiles in fig. 2 indicates that there is no endogenous peak eluted at the retention time of rofecoxib (7.5 to 8 min). For assay precision, replicates (n=6) of standard drug were analyzed to assess the inter-day and intra-day variability in the assay. The % coefficient of variation, standard deviation and recovery are shown in Table 2.

An improved simple, accurate and sensitive method has been developed for the quantitative analysis of rofecoxib. This method has been found to be specific, cost effective and has been applied for the analysis of this drug in plasma samples in bioequivalance studies.

## REFERENCES


1 Woolf, E., Fu, I., and Matuszewski, B., **J. Chromatogr. B.**, 1999, 730, 221.

2 Toyooka, T., in; Modern Derivatization Methods for Separation Sciences, John Wiley & Sons, New York, 1999,13.

3 Benson, J.R. and Hare, P.E., **Proc. Nat. Acad. Sci. USA.**, 1975, 72, 619.

4 Wolf, J.H. and Korf, J., **J. Pharm. Biomed. Analysis**, 1992, 10, 99.

5 Yoo, J.S. and McGuffin, V.L., **J. Chromatogr.**, 1992, 627, 87.

6 Farinotti, R., Siard, P., Bourson, J., Kirkiacharian, S., Valeur, B., and Mahuzier, G., **J. Chromatogr.**, 1983, 269, 81.

7 Imai, K., Fukushima, T. and Yokosu, H., **Biomed. Chromatogr.**, 1994, 8, 107.

8 Schultz, B. and Hansen, S.H., **J. Chromatogr.**, 1982, 228, 279.

9 Williams, A.T.R., Winfield, S.A. and Balloli, R.C., **J. Chromatogr.**, 1982, 240, 224.

10 Lawrence, J.F., Renault, C. and Frei, R.W., **J. Chromatogr.**, 1976, 121, 343.

11 Tsuruta, Y., Moritani. K., Date, Y. and Kohashi, K., **Anal. Sci**, 1992, 8 , 393.

12 Fujino, H. and Goya, S., **Anal. Sci**, 1992, 8 , 715.

13 Gubitz, G., Wintersteiger, R. and Frei., R.W., **J. Liq. Chromatogr.**, 1984, 7, 83.

14 Galletti, G.C. and Bocchini, P., **J. Chromatogr. A** , 1996, 729, 393.

15 Takeda, N ., Tsuji, M. and Akiyama, Y., **J. Food Hyg. Soc.** Japan, 1995, 36, 601.

16 Yu, Z., Westerlund, D. and Boss, K.S., **J. Chromatogr. B.** 1997, 689, 379.

17 Stewart, J.T. and Madhusadan, S., **J. Chromatogr. B.**, 1995, 673, 91.

18 Stewart, J.T. and Madhusadan, S., **J. Chromatogr. B.**, 1996, 682, 89.

19 Kuhlmann, O. and Krauss, G., **J. Chromatogr. Biomed. Anal.**, 1997, 16, 553.