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# Simultaneous Estimation of Metronidazole and Ofloxacin in Combined dosage form by Reverse Phase High Performance Liquid Chromatography Method

**Amit J. Kasabe*[1], Vikram V. Shitole[2], Vikram V. Waghmare[2], Vijay Mohite[3]**
**[1]Department of Pharmaceutical Chemistry,Nandha college of Pharmacy, Erode-638 052, Tamilnadu, India,**
**[2]Human Science Department,London Metro Politan University, London,**
**[3]Department of Pharmacology,Nandha college of Pharmacy, Erode-638 052, Tamilnadu, India.**

***E-mail: amit9590@gmail.com***

**Abstract:** A rapid, simple and sensitive chromatographic method (RP-HPLC) has been developed for the simultaneous estimation of Metronidazole and Ofloxacin in combined pharmaceutical dosage form (suspension). The chromatographic resolution was achieved using mobile phase acetonitrile:methanol:water at 6.5:2.5:1 (v/v). An isocratic HPLC with a single Waters 510 pumps, Waters tunable absorbance detector and µ-Bondapack C-18 column was used. The wavelength for detection was 313 nm. The flow rate was 1.0 ml/min. The validation data showed that the method is sensitive, specific and reproducible for simultaneous determination of Metronidazole (MET) and Ofloxacin (OFLOX) in combined dosage form. Calibration curves were linear from 5-100 $\mu g/ml^{-1}$($r^2$ >0.999) for MET and 5-65 $\mu g/ml^{-1}$($r^2$ >0.997) for OFLOX. Mean inter- and intra- analysis standard deviation (SD) were less than 2%. The proposed method provided an accurate and precise analysis for simultaneous estimation of MET and OFLOX by RP-HPLC method.
**Keywords:** RP-HPLC, Metronidazole, Ofloxacin, Method Validation, Pharmaceutical dosage form.

**Introduction and Experimental**: Metronidazole is an antiprotozoal and chemically it is 2-methyl-5-nitroimidazole-1-ethanol[1]. Metronidazole act as a prodrug. It is converted in anaerobic organisms by the redox enzyme pyruvate-ferredoxin oxidoreductase. The nitro group of metronidazole is chemically reduced by ferredoxin (or a ferredoxin-linked metabolic process) and the products are responsible for disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis[2]. Extensive literature survey revels that various analytical methods have been reported for the estimation of MET in single and combination form such as UV spectrophotometric[3,4], HPLC[5,6].
Ofloxacin[7] is an antimicrobial drug and chemically it is 9-fluro-2, 3 dihydro-3-methyl-10(4-methyl-1-piperazynyl-7-oxo-7H-pyrido [1, 2, 3de]-1, 4-benzoxacine-6-carboxilic acid. It acts by targeting bacterial DNA gyrase and topoisomerase IV. Extensive literature survey revels that various analytical methods have been reported for the estimation of OFLOX in single and combination form such as spectrophotometric[8,9], conductometric[10], HPLC[11-16], LC/MS/MS[17,18]. Fixed dose combination containing MET and OFLOX in suspension dosage form is available in the market and no single method is yet reported for simultaneous estimation of both these drugs. The aim of the present work is to develop a simple, rapid, accurate and selective chromatographic method for the estimation of MET and OFLOX in suspension dosage form.

**Instrument:** PerkinElmer Lambda-35 UV-Visible double beam spectrophotometer with 1cm matched quartz and Isocratic HPLC with a single Waters 510 pumps, Waters tunable absorbance detector with µ-Bondapack C-18 column.
**Chemicals and reagents**: The sample of Ofloxacin was procured from Medico Pharma, Palghar and Metronidazole from Ciron Drugs and Pharmaceuticals Ltd.India. Suspension dosage form containing MET and OFLOX combination was procured from a local pharmacy. Acetonitrile, Methanol and Orthophosphoric acid were of analytical grade.

**Preparation of solutions:**

**Selection of mobile phase:**

The pure drug of MET and OFLOX were injected into sample injector of HPLC system and run in different solvent systems, like methanol and water, acetonitrile and water, methanol acetonitrile and water were tried at different pH condition to get best condition for separation of MET and OFL. It was found that Acetonitrile: Methanol: Water gives satisfied result as compaired to other mobile phases (pH 6.5).

**Preparation of standard stock solution:**

Standard stock solution of MET and OFLOX were prepared by weighing about 10 mg of drug, dissolved in 25 ml of solvent (methanol: water 1:1) to get 5 µg/ml, 10 µg/ml, 15 µg/ml respectively of MET and OFLOX and then volume was made upto the mark with solvent to get 100µg/ml of standard stock solution of each drug.

**Preparation of working standards:**

From stock solution of metronidazole and ofloxacin 0.1 to 2 mL of solution were transferred to 10 Ml volumetric flask. The volume was made up to the mark with methanol to get a set of solutions for Metronidazole having concentration range 5,10,15,20,30,40,50,60,70,80,90,100ppm working standards solutions of both the drugs.

Preparation of calibration curves of the drug: Each of the working standard solutions were injected 6 times and the mean peak area ratio of each drug to that of standard were calculated and plotted against the concentration of drug. The regression of the concentration of each drug over the mean peak area ratio was obtained and these regression equations were used for the assay of suspension containing these drugs. The reproducibility of the method was suggested by low coefficient of variation in the peak area ratio. The precision and accuracy of the method was determined by intra and inter day variation in the mean peak area ratio for set of working solution and by the recovery study, respectively.

**Assay of formulation:**

In order to see the feasibility of proposed method for simultaneous estimation of MET and OFLOX in marketed pharmaceutical formulation, first it was tried on standard laboratory mixture (L1).

Prepare a laboratory mixture by weighing accurately 10 mg of MET and 30 mg of OFLOX and transfer to 100 ml volumetric flask and dissolved in methanol: water (1:1) and kept in ultrasonicator for 30 min .The solution was filtered through 0.45 µ membrane filter paper. This tab solution was further diluted with solvent to obtain mixed sample solution containing 5,10,20 µg/ml of MET and 15,30,60µg/ml of OFLOX respectively .Each sample solution was injected into sample injector of HPLC six times (n=6) under chromatographic condition as described above . Area of each peak was measured at 313 nm .The amount of drug present in the sample was determined from peak area of MET and OFLOX present in the pure mixture respectively (Fig 2 and Fig 4). The typical chromatogram of MET and OFLOX is shown in Fig 6. The results were statistically evaluated. Results as shown in Table- 1.

**Assay of marketed formulation (L2):**

Prepare a mixture by weighing accurately 10 mg of MET and 30 mg of OFLOX and transfer to 100 ml volumetric flask and dissolved in methanol: water (1:1) and kept in ultrasonicator for 30 min .The solution was filtered through 0.45 µ membrane filter paper. This tab solution was further diluted with solvent to obtain mixed sample solution containing 5,10,20 µg/ml of MET and 15,30,60µg/ml of OFLOX respectively .Each sample solution was injected into sample injector of HPLC six times (n=6) under chromatographic condition as described above . Area of each peak was measured at 313 nm .The amount of drug present in the sample was determined from peak area of MET and OFLOX present in the pure mixture respectively. The typical chromatogram of MET and OFLOX is shown in Fig 7. The results were statistically evaluated. Results as shown in Table- 2.

**Table-1: Assay of MET and OFLOX in laboratory mixture (L1):**

| **Sr.no** | **Amount present** | | **Amount found** | | **% of lable claim** | |
|---|---|---|---|---|---|---|
| | MET | OFLOX | MET | OFLOX | MET | OFLOX |
| 1 | 100 | 300 | 100.0 | 300.20 | 100.0 | 100.0 |
| 2 | 100 | 300 | 101.25 | 300.36 | 101.2 | 100.12 |
| 3 | 100 | 300 | 101.3 | 300.90 | 101.3 | 100.3 |
| 4 | 100 | 300 | 100.8 | 300.24 | 100.8 | 100.08 |
| 5 | 100 | 300 | 100.7 | 300.5 | 100.7 | 100.1 |

**Table-2: Assay of marketed formulation (L2):**

| Sr.no | Amount present | | Amount found | | % of lable claim | |
|---|---|---|---|---|---|---|
| | MET | OFLOX | MET | OFLOX | MET | OFLOX |
| 1 | 100 | 200 | 100.5 | 198.60 | 100.5 | 99.60 |
| 2 | 100 | 200 | 99.8 | 199.00 | 99.8 | 99.68 |
| 3 | 100 | 200 | 98.9 | 201.00 | 98.9 | 99.52 |
| 4 | 100 | 200 | 100.2 | 200.80 | 100.2 | 100.0 |
| 5 | 100 | 200 | 100.35 | 200.40 | 100.3 | 100.28 |

**Validation of Proposed method**: The proposed method was validated as per recommendations of USP[19] and ICH[20] guidelines for the parameters like recovery, precision and robestesness.

Results and Discussion: Ofloxacin (OFLOX) is a synthetic fluoroquinolone antibacterial agent, acts by inhibiting bacterial DNA gyrase enzyme which is required for DNA replication and thus causes bacterial lysis. Metronidazole is an antiprotozoal and acts by disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis. Here, an attempt has been made to develop the chromatographic method for simultaneous estimation of Ofloxacin and Metronidazole. The mean peak area of metronidazole and ofloxacin are shown in the Table-7 and Table-8. The overlain spectra of both drugs showed good absorbance at 313nm (Fig 1), hence these wavelengths were selected for estimation of OFLOX and MET. Linearity of both OFLOX and MET were obeyed Lambert's and Beer's law. The Observation table for calibration curve of MET and OFLOX is given in Table 7 and Table 8. From the data obtained it is clear that calibration curves were linear from 5-100 $\mu g/ml^{-1}$($r^2$ >0.999) for MET and 5-65 $\mu g/ml^{-1}$($r^2$ >0.997) for OFLOX (Table 9). The regression of concentration of metronidazole and ofloxacin over their peak area ratio were found to be y=61348.2 X + 43718.96 and y=360525 X – 9067999, respectively. The regressions equations were used to estimate the drugs in their formulation and in validation only.

**Recovery study:** Recovery studies were carried out by adding a known amount of standard solution of pure drugs (MET and OFLOX) ) to a preanalysed sample solution. . The study showed the result within acceptable limit of above 99% and below 101% and lower values of RSD indicates the proposed method is accurate (Table-3).

Precision: Precision studies were carried out using parameters like Intra-day and inter-day analysis Precision. The study showed the results within acceptable limit, i.e. % RSD below 2.0, indicating that the method is reproducible (Table 4 and Table 5).

**Robustesness of method**: The evaluation of robustness is considered during the development phase and was depends on the type of procedure under study. The parameters included was pH of the mobile phase, flow rate ,percentage of acetonitrile in the mobile phase .The solution containing 10µg/ml of MET and 30 µg/ml of OFLOX was injected into the sample injector of HPLC six times under different parameters as mentioned in Table-6.

**Table-3: Recovery studies of MET and OFLOX by RPHPLC method:**

| Lab mixture | Drug | Level of % recovery | Amount present (mg/tab) | Amount of std added in mg | Amt. Recovered * | % of recovery* |
|---|---|---|---|---|---|---|
| L1 | MET | 80 | 100 | 80 | 177.75 | 98.75 |
| | OF | 80 | 300 | 240 | 534 | 98.8 |
| | MET | 100 | 100 | 100 | 202 | 101.25 |
| | OF | 100 | 300 | 300 | 606 | 101.0 |
| | MET | 120 | 100 | 120 | 220.1 | 100.0 |
| | OF | 120 | 300 | 360 | 659 | 99.84 |
| L2 | MET | 80 | 100 | 80 | 178.9 | 99.3 |
| | OF | 80 | 200 | 160 | 359.9 | 99.9 |
| | MET | 100 | 100 | 100 | 199 | 99.5 |
| | OF | 100 | 200 | 200 | 400.5 | 100.1 |
| | MET | 120 | 100 | 120 | 221.0 | 100.45 |
| | OF | 120 | 200 | 240 | 439.7 | 99.93 |

***Mean of six determination (n=6)**

**Table-4: Precision: Intra –day precision:**

| Lab mixture | Drug | % Mean | ±S.D | %R.S.D. |
|---|---|---|---|---|
| L1 | MET | 99.87 | 0.53351 | 0.0548 |
| | OFLOX | 99.76 | 0.251064 | 0.0957 |
| L2 | MET | 99.89 | 0.548361 | 0.9964 |
| | OFLOX | 99.40 | 0.47606 | 0.1536 |

***Mean of six determination (n=6)**

**Table1-5: Precision: Inter –day precision:**

| Lab mixture | Drug | % Mean | ±S.D | %R.S.D. |
|---|---|---|---|---|
| L1 | MET | 101.36 | 0.743326 | 1.0425 |
| | OFLOX | 100.95 | 0.736546 | 0.0342 |
| L2 | MET | 101.35 | 0.700595 | 0.7356 |
| | OFLOX | 101.0 | 0.621718 | 0.0499 |

***Mean of six determination (n=6)**

**Table -6: Robustesness of Method:**

| SR. No | Chromatographic changes (Factors) | Retention time (tr) | | Tailing Factor(T) | |
|---|---|---|---|---|---|
| | | MET | OFL | MET | OFL |
| 1 | pH of Mobile Phase | | | | |
| | 6.5 | 1.9 | 3.8 | 1.0 | 1.5 |
| | 6.5 | 2 | 3.9 | 1.1 | 1.6 |
| | 6.6 | 2.1 | 3.99 | 1.24 | 1.55 |
| 2 | Flow rate(ml/Min) | | | | |
| | 0.9 | 2.1 | 3.8 | 1.20 | 1.59 |
| | 1.0 | 2.0 | 3.9 | 1.1 | 1.6 |
| | 1.1 | 1.9 | 3.7 | 1.0 | 1.49 |
| 3 | % of ACT | | | | |
| | 64% | 1.8 | 3.7 | 1.0 | 1.45 |
| | 65% | 2 | 3.9 | 1.1 | 1.6 |
| | 66% | 2.5 | 4 | 1.2 | 1.55 |

**Figure No. 1: Overlain UV Spectra of Metronidazole and Ofloxacin:**

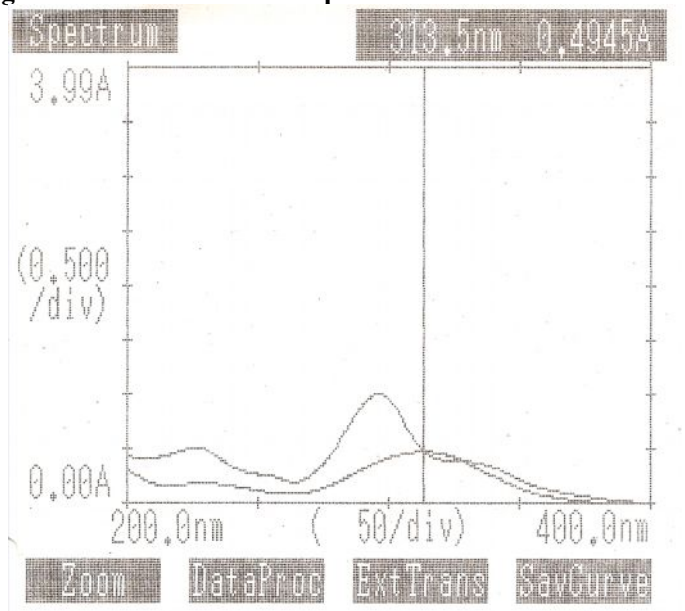


**Fig 2- Typical chromatogram of standard Metronidazole (Pure):**

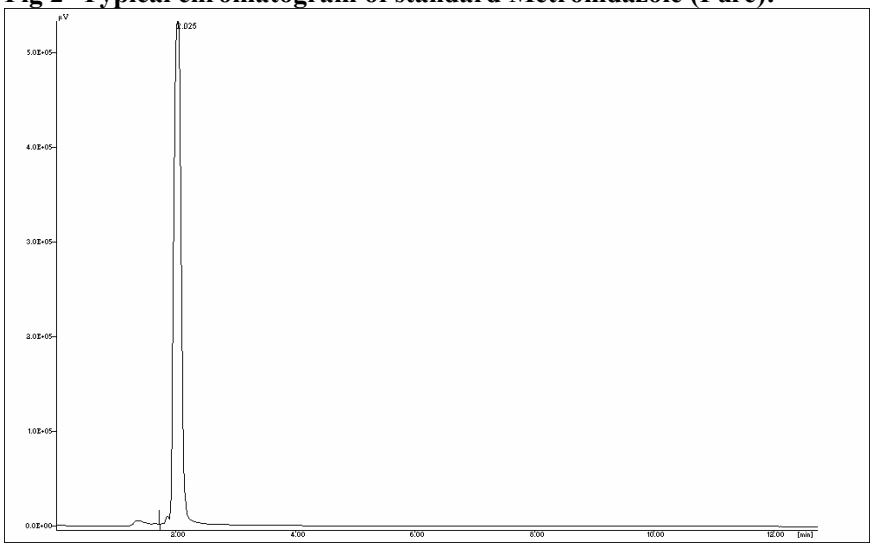
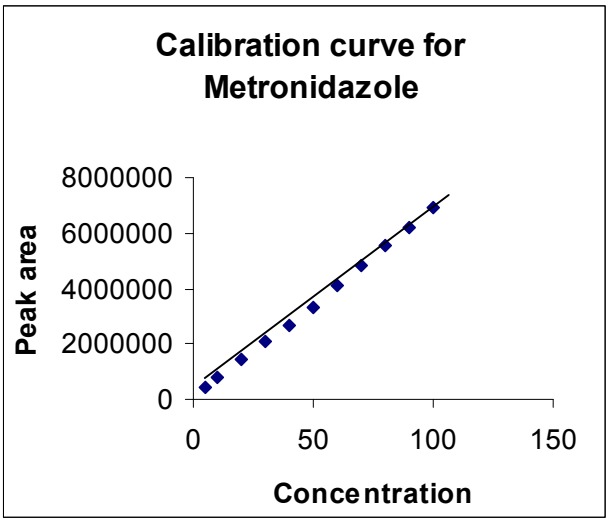


**Fig 3-Calibration curve of Metronidazole**




**Calibration curve equation,**
**$Y=360525\ X - 9067999$, $r^2=0.999$**

**Table 7 -Observation table for calibration curve of Metronidazole (n= 6):**

| Sr. no. | Concentration of Metronidazole (µg/ml) | Area under curve (AUC) |
|---|---|---|
| 1 | 5 | 439564 |
| 2 | 10 | 793474 |
| 3 | 20 | 1421828 |
| 4 | 30 | 2075394 |
| 5 | 40 | 2641732 |
| 6 | 50 | 3317200 |
| 7 | 60 | 4084532 |
| 8 | 70 | 4853857 |
| 9 | 80 | 5519686 |
| 10 | 90 | 6193724 |
| 11 | 100 | 6900352 |

**Fig 4- Typical chromatogram of standard ofloxacin:**

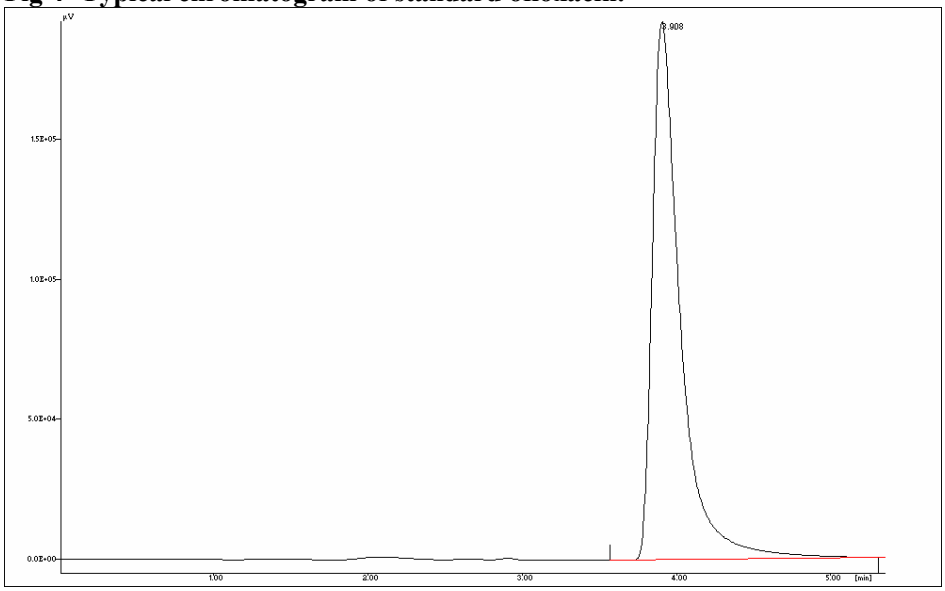


**Fig 5 -Calibration curve of Ofloxacin:**

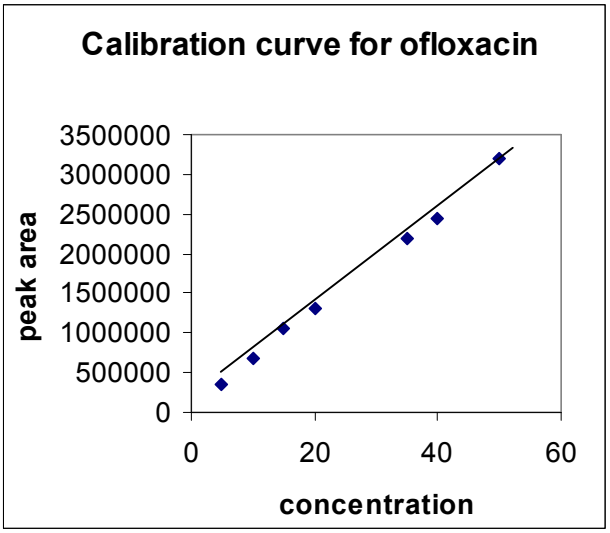



**Table 8-Observation table for calibration curve of Ofloxacin (n= 6):**

| Sr. no. | Concentration of Ofloxacin(µg/ml) | Area under curve (AUC) |
|---|---|---|
| 1 | 5 | 344784 |
| 2 | 10 | 688949 |
| 3 | 15 | 1050440 |
| 4 | 20 | 1315249 |
| 5 | 25 | 1504305 |
| 6 | 30 | 1787130 |
| 7 | 35 | 2187997 |
| 8 | 40 | 2440279 |
| 9 | 45 | 2741317 |
| 10 | 50 | 3195460 |
| 11 | 55 | 3440555 |
| 12 | 60 | 3699880 |
| 13 | 65 | 4085445 |

**Calibration curve equation**

**Y=61348.2 X + 43718.96**

**$r^2$ =0.9975**

**Table-9: Linear regression data for calibration curve of Metronidazole and Ofloxacin (n=6):**

| Name of drug | Linearity range* (µg/ml) | Slope* | Intercept* | Regression coefficient *($r^2$) |
|---|---|---|---|---|
| Metro | 5-100 | 360525 | -9067999 | 0.999 |
| Oflox | 5-65 | 61348.2 | 43718.96 | 0.997 |

**Fig 6: Typical chromatogram of MET and OFLOX present in laboratory mixture L1:**

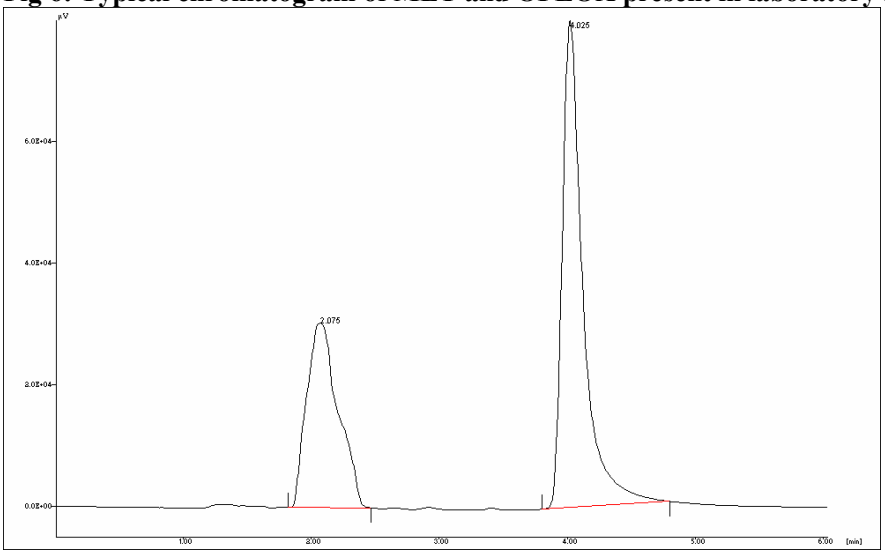
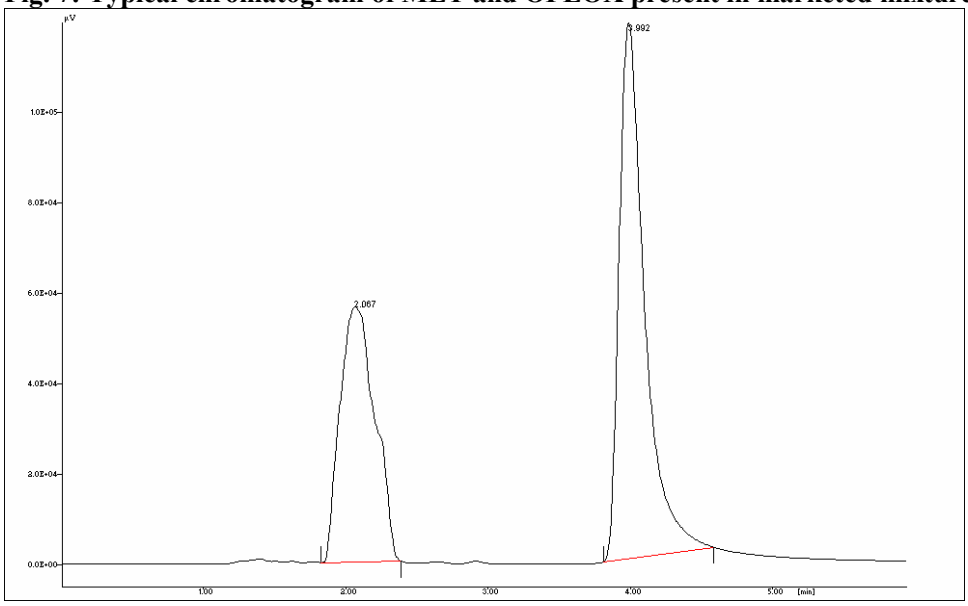


**Fig. 7: Typical chromatogram of MET and OFLOX present in marketed mixture L2:**

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