

# Analytical Methods

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**Analysis of ofloxacin and flavoxate HCl either individually or in combination *via* a green chromatographic approach with a pharmacokinetic study of ofloxacin in biological samples**

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**Abstract**

New sensitive and rapid micellar liquid chromatographic method was developed for determination of ofloxacin (OFL) and flavoxate hydrochloride (FLV) in different pharmaceutical formulations in addition to a pharmacokinetic study of OFL in human plasma and urine. The analyses were carried out on BDS Hypersil phenyl column ( $4.6 \times 250$  mm,  $5 \mu\text{m}$  particle size) using a micellar mobile phase consisting of 0.15 M SDS, 15% *n*-propanol, 0.3% triethylamine and 0.02 M orthophosphoric acid (pH 2.5) with UV detection at 325 nm. The proposed method was found to be rectilinear over the concentration range of  $2.0\text{--}40.0 \mu\text{g mL}^{-1}$  for the two drugs with lower detection limits of 0.16 and  $0.32 \mu\text{g mL}^{-1}$  for OFL and FLV, respectively. The proposed method was applied for determination of OFL and FLV in their single ingredient and co-formulated dosage forms with good percentage recoveries (99.86–99.98 %) and small standard deviation values ( $\pm 0.18 - 1.26$ ). The results of the proposed method were statistically compared with those obtained by the comparison methods revealing no significant differences in the performance of the two methods regarding accuracy and precision. To achieve high sensitivity that allows pharmacokinetic study of OFL, fluorescence detection at 290/485 nm was employed. A linear relationship was achieved over the concentration ranges of 0.01–0.10 and 0.02–0.20  $\mu\text{g mL}^{-1}$  with mean percentage recoveries of  $98.63 \pm 9.84$  and  $100.63 \pm 3.15$  % for OFL in human plasma and urine, respectively.

**Keywords:** Ofloxacin, Flavoxate HCl, MLC, Pharmaceutical Preparations, Human plasma and urine; Pharmacokinetic study.

## 1. Introduction

Ofloxacin, OFL (*7H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid, 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-, ( $\pm$ ), Fig. 1a) is a fluoroquinolone antibacterial that is used in the treatment of infections including gastro-enteritis, gonorrhea, infections of biliary-tract, joint, throat, respiratory-tract and urinary tract.<sup>1</sup> Flavoxate hydrochloride, FLV (2-(piperidin-1-yl)ethyl 3-methyl-4-oxo-2-phenyl-4H-1-benzopyran-8-carboxylate hydrochloride, Fig. 1b) is a smooth muscle relaxant with antimuscarinic effects. It is used for the symptomatic relief of pain, urinary frequency, and incontinence associated with inflammatory disorders of the urinary tract.<sup>1</sup> The combined therapy of OFL and FLV in the medicinal ratio of 1: 1 has been clinically proven as efficient treatment of urinary tract infections associated with dysuria, nocturia and suprapubic pain.

OFL is the subject of monographs in the United States Pharmacopoeia (USP<sup>2</sup>) and the British Pharmacopoeia (BP<sup>3</sup>). Both pharmacopoeias recommended non-aqueous titrimetric methods using perchloric acid as a titrant with potentiometric detection of end point for its determination in pure form and an HPLC method for its determination in ophthalmic solution. Literature survey revealed several analytical methods for determination of OFL in pharmaceutical formulations and biological matrices. A good guide to the different analytical methodologies for determination of OFL up to 2009 has been published as a monograph in the series of "*Analytical Profiles of Drug Substances and Excipients*".<sup>4</sup> Recently, more methods were reported for the analysis of OFL including spectrophotometry<sup>5</sup>, HPLC<sup>6-12</sup>, HPTLC<sup>10</sup> and capillary electrophoresis.<sup>13-17</sup>

FLV is also an official drug in the BP<sup>3</sup> that recommended a non-aqueous titrimetric method using perchloric acid as a titrant for its determination in pure form and a direct spectrophotometric method for its determination in tablets *via* measurement of its absorbance in 0.1 M HCl at 293 nm. A comprehensive review of the published analytical methods for determination of FLV up to 2001 was presented as a monograph in the series of "*Analytical Profiles of Drug Substances and Excipients*".<sup>18</sup> Some analytical methods were recently reported for determination of FLV in pharmaceutical preparations and biological fluids such as spectrophotometry,<sup>19</sup> potentiometry<sup>20</sup> and HPLC.<sup>21, 22</sup> Also, two HPLC methods have been focused on the analysis of FLV active metabolite (3-methylflavone-8-carboxylic acid) in biological fluids.<sup>23, 24</sup>

Regarding FLV/OFL pharmaceutical mixture, literature survey revealed few methods for the simultaneous assay of the two drugs. These methods include; second derivative and absorption ratio spectrophotometric methods for the simultaneous determination of the two

drugs in tablets <sup>25</sup>, and an HPLC method for their determination in spiked human plasma employing protein precipitation pretreatment and using gradient elution mode. <sup>26</sup> Another HPLC method <sup>27</sup> was applied for simultaneous determination of the two compounds in tablets using a mobile phase containing high percentage of organic solvents (acetonitrile-methanol-water, 30:20:50 v/v/v). In addition, this method suffered from poor sensitivity and narrow dynamic range (10.0-60.0  $\mu\text{g mL}^{-1}$ ). Therefore, it was essential to develop a sensitive, accurate, precise and environmentally benign method for the simultaneous determining of OFL and FLV in their combined tablets employing isocratic elution mode.

Nowadays, the society claims for the development of modern analytical methods that are not only selective and sensitive but also need to be green. In this way, the analytical chemists should develop new methodologies that don't use hazardous chemicals or solvents and generate the minimum amount of chemical wastes. Micellar liquid chromatography (MLC) represents a good substitute of conventional hydro-organic liquid chromatography in pharmaceutical and clinical analysis. MLC has several exceptional advantages including; capability of simultaneous separation of hydrophobic and hydrophilic compounds, probability of simultaneous enhancement of solvent strength and separation selectivity, reproducible retention behavior, possibility of direct injection of biological fluids, safety and cost-effectiveness. <sup>28</sup> Finally, MLC analysis meets the requirements of “*green chemistry*” concept by using eco-friendly micellar mobile phases which are less toxic and non-flammable and have lower environmental influence compared to hydro-organic mobile phases. <sup>29</sup>

Continuing the investigations in our laboratory on the utility of MLC as a green analytical methodology for the determination of many drugs in dosage forms and biological fluids <sup>30-32</sup>, this study is devoted to the development of a new MLC method for analysis of OFL and FLV in different pharmaceutical formulations in addition to *in-vivo* determination of OFL in human plasma and urine *via* direct sample injection.

## Experimental

### Instrumentation

**HPLC system:** Separation was achieved with a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-20AD delivery system, a Rheodyne injector valve, a 20  $\mu\text{L}$  loop, a SPD-20A UV-Visible detector and a RF-10A<sub>XL</sub> fluorescence detector. Mobile phases were degassed using DGU-20A5 online solvent degasser. The apparatus was interfaced to a computer for data acquisition with a CBM-20A communication bus module.

**pH-Meter:** A Consort P901 pH meter (Turnhout, Belgium) was used for pH-adjustment.

### Materials and Market Samples

- Ofloxacin pure sample (Batch No. 20110804) was kindly provided by Unipharma Co., Al Obour City, Egypt.
- Flavoxate HCl pure sample (certified purity of 99.0%) was kindly provided by Amoun Pharmaceutical Co, Cairo, Egypt.
- The following pharmaceutical formulations were purchased from local pharmacy:
  - Tarivid<sup>®</sup> film coated tablets (Batch No. 2EG012) labeled to contain 200 mg Ofloxacin/Tablet (product of Sanofi Aventis Co., Cairo, Egypt under license of Sanofi Aventis Co., Germany).
  - Oflox<sup>®</sup> eye drops (Batch No. E68342) labeled to contain 0.3 %w/v ofloxacin (product of Allergan Pharmaceuticals Ireland, Westport, Co. Mayo, Ireland).
  - Genurin S.F<sup>®</sup> 200 tablets (Batch No. 120943) labeled to contain 200 mg Flavoxate HCl/Tablet (product of Medical Union Pharmaceuticals, Abu Sultan, Ismailia, Egypt).
- The new co-formulated tablets containing OFL and FLV is not yet available in the Egyptian market, so, laboratory-prepared tablets containing 200 mg ofloxacin + 200 mg flavoxate HCl/ Tablet were formulated by thoroughly mixing 200 mg of each drug with 2 mg talc powder, 15 mg maize starch, 15 mg lactose and 10 mg magnesium stearate/ Tablet.

### Chemicals and Reagents

All solvents used were of HPLC grade, chemicals were of analytical reagent grade and high purity water was used throughout the study.

- Acetonitrile, methanol, *n*-propanol (HPLC grade) and sodium dodecyl sulfate (SDS, 99%) were purchased from Sigma-Aldrich Co. (Germany).
- Triethylamine (TEA) and orthophosphoric acid (85%, w/v) were obtained from Riedel-deHäen (Seelze, Germany).
- Cellulose acetate syringe filters with 0.45 µm pore size (Gemma Medical, Barcelona, Spain).
- Urine samples were freshly obtained from a healthy 40 years old male volunteer.
- Plasma samples were obtained from Mansoura University Hospital (MUH) and kept frozen at -20 °C until use after gentle thawing.

### Human subject

- All procedures on human subject were performed in compliance with relevant laws and institutional guidelines and it was approved by Ethics Committee of Faculty of Pharmacy, Mansoura University, Egypt. An informed consent was obtained for experimentation with human subject.
- The *in-vivo* study was carried out on a healthy non-smoker male volunteer (40 years old, 70 kg body weight) with no previous history of allergic reaction to OFL. The volunteer was instructed to withdraw from all medications for 2 weeks before administration and also during the study. The volunteer was instructed also for overnight fasting.

### Chromatographic conditions

**Column:** BDS Hypersil Phenyl column (4.6 x 250 mm, 5  $\mu$ m particle size), Thermo Electron Corporation, Runcorn, UK.

**Mobile phase:** A micellar mobile phase containing 0.15 M SDS, 15% *n*-propanol and 0.3% TEA and 0.02 M orthophosphoric acid. The pH of the mobile phase was adjusted to 2.5 with orthophosphoric acid. The mobile phase was filtered through 0.45  $\mu$ m Millipore membrane filter and sonicated for 30 min before use.

**Flow rate:** 1 mL min<sup>-1</sup>

**Detector wavelength:**

For pharmaceutical applications: UV detection at 325 nm

For biological applications: Fluorescence detection at 290/485 nm.

### Preparation of standard solutions

Standard solutions containing 200.0  $\mu$ g mL<sup>-1</sup> of each of OFL and FLV were separately prepared in methanol. OFL standard solution was diluted with methanol to obtain solutions containing 10.0 and 1.0  $\mu$ g mL<sup>-1</sup>. The standard solutions of OFL were found to be stable for one week when kept in the refrigerator at 4 °C. On the other hand, FLV standard solution was found to be highly unstable under the same conditions so it was freshly prepared daily.

### General Procedures

#### *Construction of calibration graphs*

Accurately measured volumes of OFL and FLV standard solutions were transferred into two series of 10 mL volumetric flasks to obtain final concentrations of 2.0-40.0  $\mu$ g mL<sup>-1</sup> of OFL

and FLV. The solutions were diluted to the volume with the mobile phase and mixed well. Twenty  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The average peak areas of OFL and FLV were plotted *versus* the corresponding drug concentrations ( $\mu\text{g mL}^{-1}$ ) to get the calibration graphs; alternatively, the corresponding regression equations were derived.

#### ***Analysis of laboratory-prepared mixtures containing OFL and FLV***

Aliquots of OFL and FLV standard solutions in the pharmaceutical ratio of 1:1 were transferred into a series of 10 mL volumetric flasks. The solutions were diluted to the volume with the mobile phase and mixed well. Twenty  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The mean percentage recoveries of each drug were calculated using the corresponding regression equation.

#### ***Analysis of tablets***

Ten tablets were accurately weighed, finely pulverized and thoroughly mixed. An accurately weighed amount of the powdered tablets equivalent to 20.0 mg of declared active principle was transferred into 100 mL volumetric flask. Regarding laboratory-prepared tablets containing OFL and FLV, an accurately weighed amount of the powder containing 20.0 mg of each drug were also transferred into 100 mL volumetric flask. About 90 mL of methanol was added to each flask and the powder was dispensed by sonication in an ultrasonic bath for 30 min. The solution was completed to the volume with the same solvent and filtered. The procedure for "*Construction of calibration graphs*" was followed and the nominal contents of tablets were calculated from the corresponding regression equation.

#### ***Analysis of eye drops***

An accurately measured volume of the ophthalmic solution equivalent to 300 mg OFL was transferred into 100 mL volumetric flask. The solution was made up to volume with methanol and mixed well. This solution was diluted with the same solvent to obtain a standard solution containing  $300 \mu\text{g mL}^{-1}$  of OFL. The procedure for "*Construction of calibration graphs*" was followed and the nominal contents of eye drops were calculated from the corresponding regression equation.



***Calibration graph of OFL in spiked human plasma***

Aliquots of 500  $\mu\text{L}$  of human plasma were transferred into a series of 10 mL volumetric flasks. The plasma samples were spiked with increasing volumes of OFL standard solution to obtain a final concentration range of 0.01-0.10  $\mu\text{g mL}^{-1}$ . The solutions were made up to the volume with 0.15 M SDS solution and the contents were mixed well. The solutions were filtered through 0.45  $\mu\text{m}$  cellulose acetate syringe filter then 20  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The average peak areas were plotted *versus* the drug concentration ( $\mu\text{g mL}^{-1}$ ) to obtain the calibration graph and the corresponding regression equation was derived.

***Calibration graph of OFL in spiked human urine***

Aliquots of 10  $\mu\text{L}$  of human urine were transferred into a series of 25 mL volumetric flasks and diluted with few milliliters of the mobile phase. Urine samples were spiked with increasing volumes of OFL standard solution to obtain a final concentration range of 0.02-0.20  $\mu\text{g mL}^{-1}$ . The solutions were made up to the volume with the mobile phase and the contents were mixed well. The solutions were filtered through 0.45  $\mu\text{m}$  cellulose acetate syringe filter then 20  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The average peak areas were plotted *versus* the drug concentration ( $\mu\text{g mL}^{-1}$ ) to obtain the calibration graph and the corresponding regression equation was derived.

***Pharmacokinetics study and analysis of OFL in real human plasma and urine***

Blank plasma and urine samples were collected before oral administration of a single dose of 200 mg of OFL (one Tarivid<sup>®</sup> Tablet) to a healthy over-night fasting male volunteer (40 years old), then the following procedures were followed:

***Plasma analysis***

Blood samples were collected into heparinized tubes at 0.25, 0.5, 1, 2, 3, 4, 5 hrs. Plasma samples were immediately separated by centrifugation at 3500 rpm for 15 min. Plasma samples were kept frozen at  $-20\text{ }^{\circ}\text{C}$  until analyzed after gentle thawing. 500  $\mu\text{L}$  aliquot of plasma samples were transferred into a series of 10 mL volumetric flasks, diluted to volume with 0.15 M SDS solution and mixed well. The solutions were filtered through 0.45  $\mu\text{m}$  cellulose acetate syringe filter then 20  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The concentrations of OFL in plasma samples

were calculated from the previously derived regression equation. The concentration-time profile curve for OFL in plasma was plotted.

### ***Urine analysis***

Urine samples were collected at appropriate time intervals post-dose after 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14 and 22 hrs. The samples were kept frozen at -20 °C until analyzed after gentle thawing. Ten  $\mu\text{L}$  aliquot of urine samples were transferred into a series of 25 mL volumetric flasks, diluted to volume with the mobile phase and mixed well. The solutions were filtered through 0.45  $\mu\text{m}$  cellulose acetate syringe filter then 20  $\mu\text{L}$  aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The concentrations of OFL in urine samples were calculated from the previously derived regression equation. The excretion curve of OFL in urine was constructed.

### **Results and Discussion**

The current study represents a fast, reliable and environmentally benign method for the determination of FLV and OFL in their newly marketed co-formulated tablets in addition to their single ingredient dosage forms. One of the main applications of MLC is the possibility of direct sample injection of biological materials into the column due to the ability of micellar aggregates to dissolve sample proteins and other components. Thus, the proposed MLC method was applied for biological monitoring and pharmacokinetic study of OFL in human plasma and urine adopting fluorescence detection to achieve high sensitivity. Regarding FLV, it is absorbed from the gastrointestinal tract and rapidly metabolized in plasma to 3-methylflavone-8-carboxylic acid,<sup>1</sup> about 50-60% of the dose being excreted in the urine within 24 hrs as the metabolite.<sup>1</sup> FLV intact drug is not detected in human plasma or urine after oral ingestion.

The proposed MLC method has the advantages of being simple, green and eco-friendly due to the use of small amount of organic solvents compared to conventional hydro-organic LC methods. Also, the presence of micelles stabilizes the mobile phase and prevents vaporization of the organic solvent, so, it is safe to both the analyst and the environment.<sup>29</sup>

### **Method Development and Optimization of Chromatographic Conditions**

A comprehensive study was conducted to select the most efficient parameters for the analysis. To achieve the best chromatographic conditions, the stationary phase type, detection wavelength and mobile phase composition were carefully investigated. The results of the optimization study are summarized below.

### ***Choice of column***

Three columns were tested for performance investigation including:

1. CLC Shim-pack C<sub>8</sub> column (250 mm×4.6 mm, 5 µm particle size), Shimadzu Corporation, Japan.
2. CLC Shim-pack CN column (150 mm×4.6 mm, 5 µm particle size), Shimadzu Corporation, Japan.
3. BDS Hypersil phenyl column (250 mm×4.6 mm, 5 µm particle size), Thermo Electron Corporation, Runcorn, UK.

FLV was strongly retained on the C<sub>8</sub> column so that, no peaks appeared even after 40 min. This is attributed to the high lipophilicity of FLV where it has Log  $P_{\text{(octanol/water)}}$  of 4.9.<sup>33</sup> Consequently, more polar columns including phenyl and cyanopropyl columns were tested to decrease the retention of FLV. A Phenyl column was found to be superior to the cyanopropyl one since it gave symmetrical, well resolved peaks of OFL and FLV within a reasonable analytical run time (about 8 min), while the cyanopropyl column yields asymmetrical peak of FLV.

### ***Choice of appropriate detection wavelength***

The absorption and fluorescence behavior of OFL and FLV were investigated for the choice of optimum detection wavelength. Online fluorescence scan analysis revealed highest sensitivity for detection of OFL at 290/485 nm. On the other hand, investigation of the fluorescence behavior of FLV revealed no native fluorescence. Hence, UV detection was employed to allow simultaneous determination of the two compounds in pharmaceuticals while fluorescence detection was applied for determination of OFL in biological fluids. A wavelength of 325 nm was selected as the optimum detection wavelength since it allowed the simultaneous determination of the two compounds in their pharmaceutical ratio of 1:1 with satisfactory sensitivity. While fluorescence detection was employed at 290/485 nm for biological analysis of OFL.

### ***Effect of pH of the mobile phase***

In order to optimize the separation process, pH of the mobile phase was investigated over the range of 2.5-5.6. The retention of the two compounds was scarcely affected by the change in the pH. The pH of the mobile phase was kept at pH 2.5 throughout the study. At this pH, the ionization of silanol groups of the stationary phase is reduced thus, minimizing its interaction

with OFL and FLV which are present as cationic species since  $pK_a$  values of OFL are 6.05 and 8.22<sup>34</sup> and that of FLV is 7.3.<sup>33</sup> Results of pH optimization study are presented in Table 1.

### ***Effect of SDS concentration***

Molar concentration of SDS was studied over the range of 0.10-0.15 M. In all mobile phases studied the separation was achieved, while a second step to be taken into consideration was to obtain a minimum analysis time with satisfactory resolution. It was found that an increase in SDS concentration produced a decrease in retention factor ( $k'$ ) of the two compounds (Table 1). A mobile phase containing 0.15 M SDS was finally chosen as the optimum one taking in consideration the total analysis time, peak symmetry, resolution and column efficiency.

### ***Effect of type and concentration of organic modifier***

The most important drawback of MLC is the decrease of chromatographic efficiency due to poor wetting of the stationary phase and poor mass transfer.<sup>28, 29</sup> To improve the chromatographic efficiency of MLC, it has been proposed to add a small amount of organic modifier. Four mobile phases containing *n*-propanol (15% v/v), methanol (18% v/v), pentanol (7% v/v) and tetrahydrofuran (15% v/v) were tested to select the best organic modifier. The results obtained indicated that *n*-propanol is the organic modifier of choice since it gave symmetrical peaks with best resolution. Despite that, all other tested organic modifiers could separate the two compounds but pentanol resulted in lower resolution and inferior sensitivity relative to *n*-propanol, while methanol yielded broad peaks of OFL and FLV. On the other hand, using tetrahydrofuran as organic modifier resulted in poor sensitivity of OFL and FLV.

The % concentration of *n*-propanol was varied over the range of 10-15% v/v. The retention of OFL and FLV decreased with the increase in the organic modifier concentration. A concentration of 15% v/v of *n*-propanol was chosen as the optimal concentration, where it provided a good compromise of peak symmetry and sharpness; resolution factor and analysis time (Table 1).

After these experimental investigations, the analyses were performed using a phenyl column and mobile phase consisting of 0.15 M SDS-15% *n*-propanol-0.3% TEA-0.02 M  $H_3PO_4$  at pH 2.5. Figure 2 represents a typical chromatogram showing good resolution of OFL and FLV ( $R_s = 3.2$ ) under the optimum chromatographic conditions. Despite the large difference in the polarity of the two compounds ( $\log P_{(octanol/water)}$  of FLV and OFL are 4.9<sup>33</sup> and -0.48,<sup>34</sup>

respectively), the use of a micellar mobile phase allowed their simultaneous determination in an isocratic mode.

### Method Validation

The proposed method was validated according to ICH Q2 (R1) Guidelines.<sup>35</sup> The investigated criteria included linearity, range, limit of quantification, limit of detection, accuracy, precision, specificity, robustness, system suitability and stability of samples and mobile phases.

#### *Linearity and range*

Calibration curves were constructed, using the average areas of the chromatographic peaks, over the range of 2.0-40.0  $\mu\text{g mL}^{-1}$  for both of OFL and FLV. The validity of the method was proven by statistical evaluation of the regression line.<sup>36</sup> The fairly small values of the standard deviation of the residuals ( $S_{y/x}$ ), slope ( $S_b$ ) and intercept ( $S_a$ ), and the % relative error indicate low scattering of the calibration points around the regression line (Table 2).

#### *Limit of quantification (LOQ) and limit of detection (LOD)*

The limit of quantification (LOQ) and limit of detection (LOD) were determined according to ICH Q2 (R1) guidelines<sup>35</sup> adopting the approach of standard deviation of the response and the slope according to the following equations:

$$\text{LOQ} = 10S_a/b$$

$$\text{LOD} = 3.3S_a/b$$

Where,  $S_a$  is the standard deviation of the intercept of regression line and  $b$  is its slope.

The obtained results are also presented in Table 2.

#### *Accuracy*

To test the accuracy of the proposed method, it was applied to the determination of pure samples of OFL and FLV over the concentration range of 2.0-40.0  $\mu\text{g mL}^{-1}$ . The results obtained were in good agreement with those obtained by the comparison HPLC method.<sup>27</sup> Statistical evaluation of the results<sup>36</sup> using the Student  $t$ -test and the variance ratio  $F$ -test revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 3).

### ***Precision***

#### ***Repeatability***

Repeatability (intra-day precision) of the proposed method was tested by triplicate analysis of three different concentrations of OFL and FLV in pure form for three successive times. The results are summarized in Table 4.

#### ***Intermediate precision***

Intermediate precision (inter-day precision) was evaluated through triplicate analysis of three different concentrations of the two drugs on three successive days. The results obtained are abridged also in Table 4.

The data presented in Table 4 indicate high precision of the developed method. Good values of the average percentage recoveries and small values of % RSD indicate the high precision of the proposed method.

### ***Specificity***

The specificity of the proposed method was proven by its ability to determine OFL and FLV in their tablets without interference from common excipients and additives. In addition, the proposed method is specific for OFL in its eye drops without interference from common additives and benzalalkonium chloride preservative which is undetectable at the detection wavelength, 325 nm.

For biological applications, interferences encountered from endogenous components and proteins were assessed by analyzing blank plasma and urine in addition to plasma and urine samples spiked with OFL. Moreover, plasma and urine samples obtained from volunteers given OFL (200 mg Tarivid® tablet) orally were analyzed. No interfering peaks were observed in the obtained chromatograms indicating the specificity of the developed method. A very small protein peak was detected a head of the chromatogram due to dilution of plasma and urine samples with the mobile phase with dilution factors of 1:20 and 1:2500, respectively. Such dilution factor minimizes the width of protein band thus; diminishes the interference from proteins and other endogenous components.

### ***Robustness***

For evaluation of the robustness of the proposed method, one chromatographic parameter was changed while keeping all others unchanged. The studied variables included; pH of the mobile phase ( $2.5 \pm 0.2$ ), molar concentration of SDS ( $0.15 \pm 0.001$  M) and % concentration of

*n*-propanol ( $15 \pm 0.5$  mL v/v). These minor changes did not affect the separation and resolution of OFL and FLV.

### ***System suitability test parameters***

To ascertain the suitability of the developed method, system suitability tests were performed using the working standard solutions of OFL and FLV. Resolution ( $R_s$ ), theoretical plate number ( $N$ ), capacity factor ( $k'$ ) and relative retention ( $\alpha$ ) were measured as the criteria for system suitability testing. Table 5 represents the final system suitability test parameters.

### ***Stability of standard solutions and mobile phase***

Stability of the standard solutions of OFL and FLV was assessed by quantification of the two compounds and comparison to freshly prepared standard solutions. No significant change was observed in OFL standard solution response over a period of 2 days relative to freshly prepared standard solution. Regarding FLV, it was found to be labile to degradation, where 5 % degradation of FLV sample was observed after standing for 6 hrs at laboratory temperature with the appearance of a well separated degradation product at 5.9 min (Fig. 3). Moreover, complete degradation of FLV was observed after storage at 4 °C for 7 days, as revealed by disappearance of its peak and appearance of the peak of its degradation product at 5.9 min. So, to prevent hydrolysis, it is recommended to freshly prepare FLV standard solution daily. Similarly, stability of the mobile phase was checked. Regarding mobile phase stability, the obtained results proved that, the mobile phase used is stable up to 2 days when kept in the refrigerator at 4 °C.

### ***Applications***

#### ***Quality control of OFL and FLV in pharmaceutical formulations***

The proposed method was successfully applied for simultaneous determination of OFL and FLV in laboratory-prepared mixtures in the medicinal ratio of 1:1, and consequently it was applied for simultaneous determination of the two drugs in their co-formulated tablets. Moreover, the proposed method was applied for the determination of OFL in its eye drops and tablet formulations, in addition to the determination of FLV in its single ingredient tablet formulation.

Statistical analysis of the results obtained by the proposed method and those given by the comparison HPLC method<sup>27</sup> was performed using Student's *t*-test and the variance ratio *F*-test.

<sup>36</sup> As illustrated in Table 3, the calculated *t*- and *F*- values did not exceed the theoretical ones,

indicating no significant difference in the performance of the proposed and comparison methods regarding accuracy and precision, respectively. Figure 4 shows the chromatograms obtained for analysis of different formulations containing the two studied compounds.

### Pharmacokinetic study of OFL

MLC appears as a promising technique for the direct injection of physiological samples. Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs. Proteins are solubilized and washed harmlessly away. The use of MLC for biological analysis of pharmaceuticals decreases the time and costs of analysis with increased sample output.<sup>28</sup> These facts encouraged us to apply the developed MLC method for determination of OFL in human plasma and urine.

OFL is rapidly absorbed after oral administration. A small amount of the dose is metabolized to desmethyl OFL and N-oxide OFL. Excretion is mainly *via* the kidneys with around 80% of the dose being excreted as the unchanged drug in urine over 24 to 48 hrs.<sup>33</sup>

In the current study, pharmacokinetic investigation of OFL was performed following the oral administration of a single dose of Tarivid<sup>®</sup> tablet containing 200 mg of OFL to a healthy male volunteer (40 years old). No treatment of plasma or urine samples rather than dilution and filtration was made before injection of samples into the chromatograph. When diluting plasma samples with the mobile phase some precipitation was observed. This is probably due to the use of 15 % v/v n-propanol as organic modifier in the mobile phase. So, to avoid this precipitation, dilution of plasma was carried out with 0.15 M SDS.

Results for determination of OFL in spiked human plasma and urine are depicted in Table 6. Figure 5 represents typical chromatograms for OFL in human plasma and urine. Figure 6A shows the plasma concentration-time profile curve for OFL. A maximum plasma concentration ( $C_{max}$ ) of  $0.87 \mu\text{g mL}^{-1}$  was attained 30 min ( $T_{max}$ ) after the oral administration of OFL. The maximum concentration ( $C_{max}$ ) excreted in urine 1 hr after oral administration was found to be  $330 \mu\text{g mL}^{-1}$ . These results are in good conformity with the data found in the literature.<sup>12</sup> Figure 6B shows urinary elimination curve of OFL following 200 mg oral dose. The obtained results indicate that the proposed procedure is particularly useful for pharmacokinetic studies with patients using small volume of urine.

Comparing the proposed MLC method with previously reported MLC method for a number of quinolones including OFL,<sup>12</sup> the proposed method is superior for quantitation of OFL in biological fluids since it is more time saving ( $t_R$  of OFL = 4.9 min *versus* 14 min in the reported method<sup>12</sup>), also, our method is applicable for therapeutic drug monitoring in both



human plasma and urine where the reported one was applied only for determination of OFL in urine and no study was conducted on plasma. Despite LC/MS methods <sup>6, 8</sup> provide high sensitivity and selectivity but the instrument is very expensive, not available in many laboratories and requires expertise to deal with.

### **Conclusion**

As a conclusion, a new green, simple and reproducible HPLC method has been developed for the determination of OFL and FLV in drug substance and pharmaceutical formulations. The developed MLC method was applied for clinical pharmacokinetic study of OFL in human plasma and urine following single 200 mg oral dose without any pretreatment steps. The validation data of the proposed method demonstrate good precision and accuracy.

### **Authors' Contribution**

R. N. El-Shaheny suggested the idea and planned the study, carried out the experimental work, performed statistical analysis of the data and wrote the manuscript; N. El-Enany and F. Belal supervised the work and revised the manuscript. All authors approved the final article.

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**Table 1**

Results of optimization study for separation of FLV and OFL.

Parameter		No of theoretical plates ( <i>N</i> )		Retention factor ( <i>k'</i> )		Resolution ( <i>R<sub>s</sub></i> )	Selectivity factor ( <i>α</i> )
		OFL	FLV	OFL	FLV		
pH of mobile phase	2.5	1892	1023	0.61	1.34	2.04	2.20
	3.0	1689	804	0.65	1.44	1.99	2.22
	4.0	1850	1117	0.61	1.30	2.19	2.13
	5.6	1596	1065	0.66	1.33	1.92	2.02
SDS Conc. (M)	0.10	1623	548	1.39	2.99	3.50	2.15
	0.12	1374	616	1.17	2.49	3.30	2.13
	0.13	1323	507	1.07	2.29	2.99	2.14
	0.15	1811	1031	1.03	2.17	2.98	2.11
% n-propanol	10	1411	879	531	1.03	2.17	2.98
	12	1176	376	0.93	1.96	2.48	2.11
	15	1825	1020	0.65	1.44	3.23	2.22

**Table 2**

Analytical performance data for the proposed method

Parameter	Results	
	OFL	FLV
<b>Concentration range</b> ( $\mu\text{g/ml}$ )	2.0-40.0	2.0-40.0
<b>Limit of detection (LOD)</b> ( $\mu\text{g/ml}$ )	0.16	0.32
<b>Limit of quantification (LOQ)</b> ( $\mu\text{g/ml}$ )	0.49	0.98
<b>Correlation coefficient (r)</b>	0.9999	0.9999
<b>Slope</b>	$5.3 \times 10^4$	$4.5 \times 10^4$
<b>Intercept</b>	$-6.0 \times 10^3$	$8.59 \times 10^3$
<b>Standard deviation of the residuals (<math>S_{y/x}</math>)</b>	$5.7 \times 10^3$	$9.80 \times 10^3$
<b>Standard deviation of the intercept (<math>S_a</math>)</b>	$2.6 \times 10^3$	$4.45 \times 10^3$
<b>Standard deviation of the slope (<math>S_b</math>)</b>	$1.7 \times 10^2$	$2.89 \times 10^2$
<b>% RSD</b>	0.67	0.90
<b>% Error</b> ( $\% \text{RSD}/\sqrt{n}$ )	0.27	0.37

**Table 3**

Application of the proposed and comparison methods to the determination of OFL and FLV in pure and different dosage forms.

Matrix	Mean % Found <sup>a</sup> ± SD		<i>t</i> -value <sup>b</sup>	<i>F</i> -value <sup>b</sup>
	Proposed method <sup>a</sup>	Reference method <sup>27</sup>		
Pure form				
OFL	100.16 ± 0.67	99.94 ± 0.45	0.504 (2.365)*	2.194 (5.786)*
FLV	99.76 ± 0.90	100.15 ± 1.04	0.591 (2.365)*	1.339 (5.786)*
Pharmaceutical formulations				
1-Tarivid <sup>®</sup> Tablets (200 mg OFL/Tab.)	99.98 ± 0.18	100.20 ± 1.41	0.300 (2.776)*	12.742 (19.00)*
2-Oflox eye drops (0.3% OFL)	99.95 ± 0.40	99.77 ± 1.66	0.193 (2.776)*	16.537 (19.00)*
3-Genurin S.F <sup>®</sup> Tablets (200 mg FLV/Tab.)	99.86 ± 0.99	100.20 ± 1.45	0.346 (2.776)*	2.109 (19.00)*
4-Laboratory-prepared tablets (200 mg OFL+ 200 mg FLV/Tab.)				
OFL	99.89 ± 1.08	100.17 ± 1.16	0.310 (2.776)*	1.165 (19.00)*
FLV	99.87 ± 1.26	99.77 ± 1.59	0.080 (2.776)*	1.587 (19.00)*

<sup>a</sup> Each result is the average of three separate determinations.

\* Values between parenthesis are the tabulated *t* and *F* values at *P*=0.05.<sup>36</sup>



Table 4

Precision data for determination of OFL and FLV in pure form.

OFL			
Conc. (µg/mL)	% Found ± SD	% RSD	% Error
<b>Intra-day precision</b>			
5.0	99.13 ± 1.02	1.03	0.59
10.0	99.02 ± 0.91	0.92	0.53
30.0	99.96 ± 1.00	1.00	0.60
<b>Inter-day precision</b>			
5.0	98.6 ± 0.90	0.90	0.50
10.0	101.22 ± 1.33	1.31	0.76
30.0	99.15 ± 0.65	0.65	0.38
FLV			
Conc. (µg/mL)	% Found ± SD	% RSD	% Error
<b>Intra-day precision</b>			
5.0	99.55 ± 1.47	1.48	0.85
10.0	99.96 ± 0.98	0.98	0.57
30.0	100.76 ± 0.36	0.35	0.20
<b>Inter-day precision</b>			
5.0	100.61 ± 1.38	1.37	0.79
10.0	100.36 ± 1.40	1.40	0.81
30.0	101.01 ± 1.04	1.03	0.59

**Table 5**  
Final system suitability parameters for the proposed method

Parameter	OFL	FLV
No of theoretical plates, $N$	1892	1022
Retention factor, $k'$	0.61	1.34
Selectivity factor, $\alpha$		2.2
Resolution, $R_s$		3.2

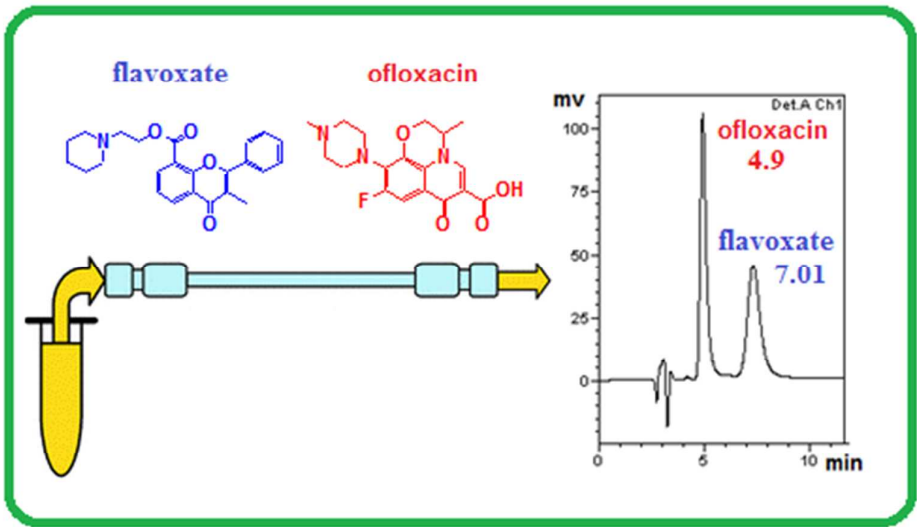
**Table 6**

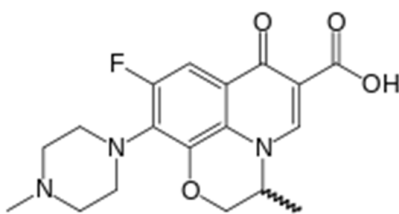
Application of the proposed method to the determination of OFL in spiked human plasma and urine

Parameter	Amount added ( $\mu\text{g/ mL}$ )	Amount found ( $\mu\text{g/ mL}$ )	% Recovery <sup>a</sup>
Spiked urine	0.02	0.020	100.00
	0.05	0.043	86.00
	0.10	0.110	110.00
	0.20	0.197	98.50
Mean found (x')	98.63		
$\pm$ SD	9.84		
% RSD	9.98		
% Error	4.92		
Spiked plasma	0.01	0.010	100.00
	0.02	0.021	105.00
	0.04	0.039	97.50
	0.10	0.100	100.00
Mean found (x')	100.63		
$\pm$ SD	3.15		
% RSD	3.13		
% Error	1.57		

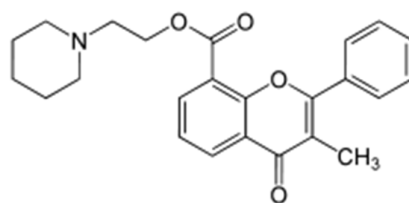
<sup>a</sup> Each result is the average of three separate determinations.

An eco-friendly MLC method was developed and validated for determination of ofloxacin and fleroxate either individually or in combined tablets in addition to biological analysis of ofloxacin.





(a)



. HCl

(b)

Fig.1 Chemical structure of (a) Ofloxacin and (b) Flavoxate hydrochloride

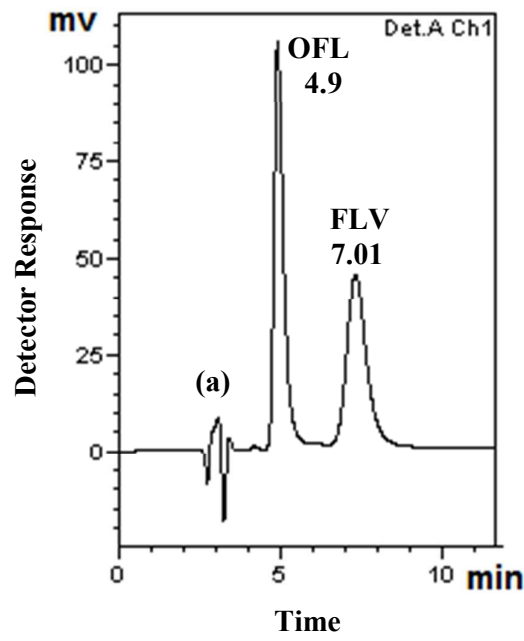


Fig. 2 Representative chromatogram showing good resolution of OFL (40  $\mu\text{g/mL}$ ) and FLV (40  $\mu\text{g/mL}$ ).

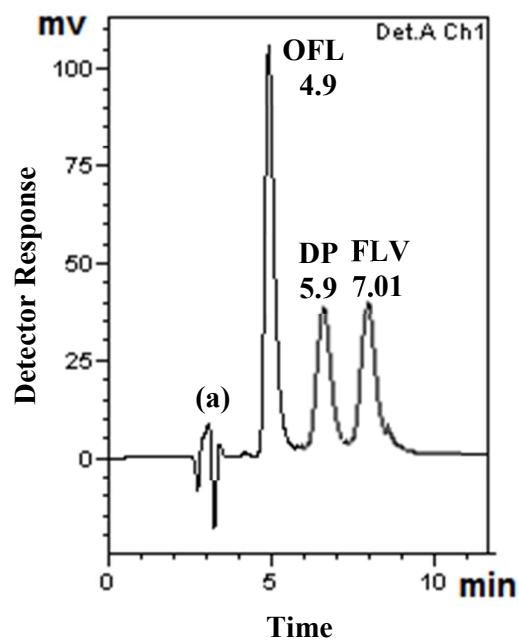


Fig. 3 Representative chromatogram showing laboratory-prepared mixture of OFL (40  $\mu\text{g/mL}$ ) and FLV (40  $\mu\text{g/mL}$ ) after standing for 6 hrs at laboratory temperature, where DP is the degradation product of FLV.

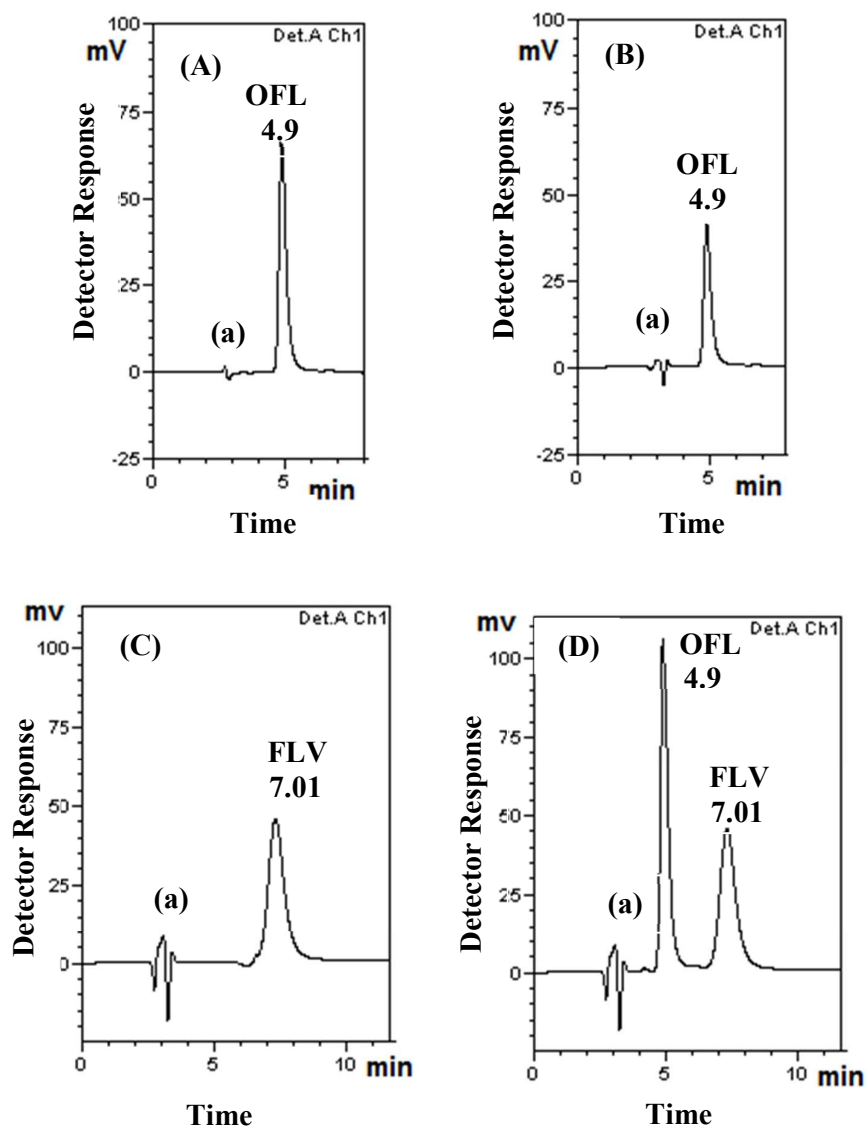


Fig. 4 Representative chromatograms showing:

- (A) OFL (30 µg/mL) in eye drops.
- (B) OFL (20 µg/mL) in tablets.
- (C) FLV (40 µg/mL) in tablets.
- (D) OFL (40 µg/mL) and FLV (40 µg/mL) in laboratory-prepared co-formulated tablets.



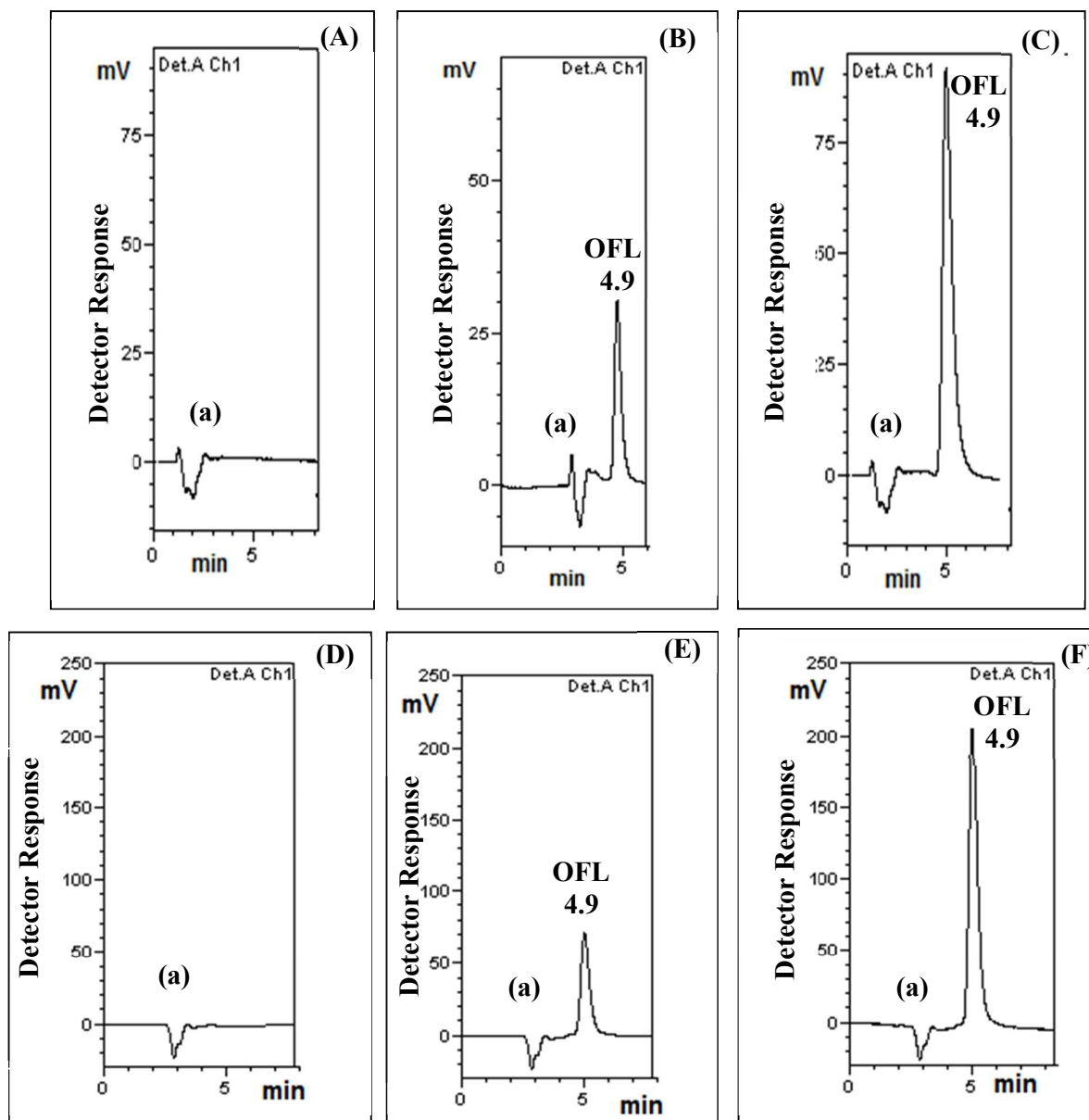


Fig. 5 Representative chromatograms showing:

- (A) Blank plasma.
- (B) Plasma sample spiked with OFL (0.01  $\mu\text{g/mL}$ ).
- (C) OFL in real human plasma 30 min after 200 mg oral dose.
- (D) Blank urine.
- (E) Urine sample spiked with OFL (0.05  $\mu\text{g/mL}$ ).
- (F) OFL excreted in urine as unchanged drug 1 hr after 200 mg oral dose.

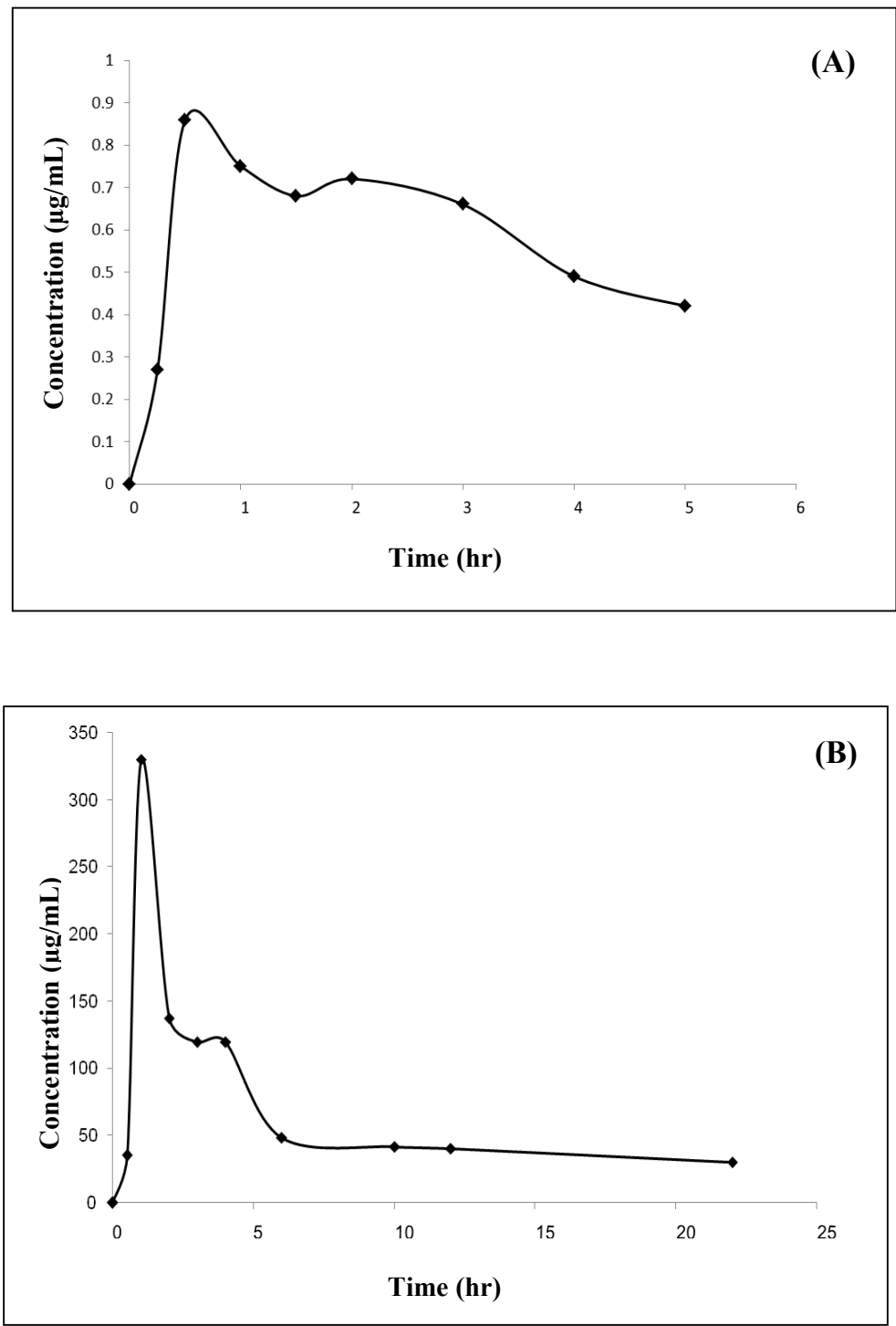


Fig. 6  
(A) Concentration-time profile curve of OFL in plasma of a healthy volunteer after 200 mg oral dose.  
(B) Excretion curve of OFL in urine of a healthy volunteer after 200 mg oral dose.