Journal of Applied Pharmaceutical Science Vol. 6 (07), pp. 001-011, July, 2016
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2016.60701
ISSN 2231-3354 (cc) BY-NC-SA

Development and validation of a stability indicating HPLC-diode array-fluorescence method for the determination of meclofenoxate hydrochloride and *p*-chlorophenoxyacetic acid

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ARTICLE INFO

Article history:
Received on: 30/04/2016
Revised on: 14/05/2016
Accepted on: 21/06/2016
Available online: 28/07/2016

Key words:
Meclofenoxate;*p*-chlorophenoxyacetic acid; stability indicating HPLC; kinetics; Dissolution; fluorimetric detection.

ABSTRACT

The present work deals with the development and validation of a stability indicating HPLC-diode array-fluorescence method (HPLC-DAD-FL) for the determination of meclofenoxate hydrochloride (MFX) and its hydrolytic degradation product *p*-chlorophenoxyacetic acid (*p*CPA). The chromatographic separation was achieved using a reversed phase Zorbax Eclipse SB-C18 column (250 x 4.6 mm i.d., 5 µm particle size), a mobile phase composed of 20 mM phosphate buffer, adjusted to pH 3, and acetonitrile (65: 35, v/v); and isocratic flow at a rate of 1 mL min^{-1}.The detection was carried out using a diode array detector (DAD) at 225 nm and a fluorescence detector (FL) at $\lambda_{ex/em}$, 225/310 nm. The linear dynamic ranges were found to be 0.5-100 and 0.25-100 µgmL^{-1} (DAD); and 0.05-20 and 0.01-8 µg mL^{-1} (FL) for MFX and *p*CPA, respectively. Detection limits were 0.05 and 0.04µgmL^{-1} (DAD); and 0.005 and 0.001 µg mL^{-1} (FL) for MFX and *p*CPA, respectively. The investigated method was applied to the assay of MFX in tablets and vials dosage forms and also to the kinetics stability studies of MFX in different media. Further, the dissolution rate of the tablets was examined using the proposed method.

INTRODUCTION

Meclofenoxate hydrochloride (MFX), (4-chlorophenoxy)-acetic acid 2-(dimethylamino) ethyl ester hydrochloride (Sweetman, 2009), is a cerebral stimulant. It acts as a nootropic agent used to treat symptoms of senile dementia and alzheimer's disease (Marcer and Hopkins, 1977; Wood and Peloquin, 1982).

MFX is not official in the British Pharmacopoeia (BP) and the United States Pharmacopeia (USP). Several analytical techniques have been reported for the analysis of MFX, including: HPLC methods (Cooke *et al.*, 1979; Tatsuhara and Tabuchi, 1980; Yoshioka *et al.*, 1982; Yoshioka *et al.*, 1983; Ohta *et al.*, 1986; El-Bardicy *et al.*, 2007 a; Ling-yun *et al.*, 2007), voltammetry using novel sensor based on gold nano-particles (Li *et al.*, 2012), potentiometry (El-Bardicy *et al.*, 2007 b;El-Nashar *et al.*, 2012), resonance Rayleigh scattering method (Hu *et al.*, 2010), proton magnetic resonance spectroscopy (Shoukrallah *et al.*, 1990; Zhang *et al.*, 2016), spectrophotometry (Fecko, 1973; Araman *et al.*, 1992), radiochemistry (Cecal *et al.*, 1983) and microcalorimetry (Otsuka *et al.*, 1994). MFX is an ester drug, it is highly susceptible to hydrolysis in aqueous solution to give hydrolytic products, *p*-chlorophenoxyacetic acid (*p*CPA) and N, N-dimethyl ethanol amine (Cooke *et al.*, 1979). Several studies have handled the stability of MFX in aqueous and/or non-aqueous solutions (Cooke *et al.*, 1979; Tatsuhara and Tabuchi, 1980; Yoshioka *et al.*, 1982; Yoshioka *et al.*, 1983; Ohta *et al.*, 1986; Araman *et al.*, 1992; Otsuka *et al.*, 1994; El-Bardicy *et al.*, 2007 a; El-Bardicy *et al.*, 2007 b; El-Nashar *et al.*, 2012), however some of the results of these investigations are contradicted. The inconsistency around stability data was the motivator for the present work. The present work describes an HPLC-diode array-fluorescence (HPLC-DAD-FL) method for the determination of MFX and *p*CPA.

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To our knowledge, no HPLC-fluorescence detection method has been reported for the determination of MFX or *p*CPA. The first target of this work was to establish stability indicating assay method, so the method was applied to study the forced hydrolytic, oxidative and photolytic degradation of MFX. MFX showed good resolution from all degradation products. It should be noted that the other reported methods have considered only the hydrolytic degradation.

Another target was to apply the developed method to the kinetic stability study of MFX, to investigate its half-life time in aqueous and non aqueous solutions to give enough information about the most appropriate solvent for the stability of MFX standard solutions and the best pH for analytical method optimization. Also, the method was applied to the determination of MFX and its degradation product, *p*CPA in pharmaceutical formulations and for MFX tablets dissolution testing.

MATERIALS AND METHODS

Instrumentation

The HPLC equipment was Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) (quaternary pump, vacuum degasser) with auto-sampler (G13298 1260 series ALS) and two detectors: diode array and multiple wavelength detector (G1315 C/D and G1365 C/D); and a fluorescence detector (G1321C 1260 series FLD) connected to a computer loaded with Agilent ChemStation Software. The column used was Zorbax Eclipse SB-C18 (250 x 4.6 mm i.d., 5 µm particle size) Agilent. The flow rate was maintained at 1 mL min^{-1} and the injection volume was 20 µL. The mobile phase was prepared daily, filtered through a 0.45 µm membrane filter.

The photo-degradation was carried out using an ultra violet lamp of the Horizontal Laminar Flow Clean Work Bench modelNU-201 and NU-201(E).

The dissolution testing of MFX Lucidril® tablets was performed on a six station dissolution apparatus, Hanson Research Corp., Northridge California,USA.

Materials and reagents

Authentic sample of MFX,98% was purchased from Alfa Aesar, a Johnson Matthey Company, Karlsruhe, Germany. Lucidril® tablets labeled to contain 250 mg MFX per tablet and Luciforte® vials labeled to contain 500 mg MFX per vial, both are manufactured by Minapharm, Egypt and purchased from local market. HPLC grade acetonitrile and methanol were purchased from LAB- SCAN Analytical Sciences, Poland. HPLC grade *o*-phosphoric acid (LOBA chemie PVT-LTD. Mumbai, India) was used. Disodium hydrogen phosphate, sodium hydroxide, hydrochloric acid, 30% hydrogen peroxide were of analytical grade. High purity distilled water was used.

Chromatographic conditions

The chromatographic separation was performed using a reversed phaseZorbax Eclipse SB-C18 column (250 x 4.6 mm i.d., 5 µm particle size) Agilent maintained at 25°C, eluted with mobile phase at a flow rate of 1 mL min^{-1}.The mobile phase consisted of 20mM phosphate buffer adjusted to pH 3 (using *o*-phosphoric acid) and acetonitrile (65: 35, v/v), filtered prior to use through 0.45 µm nylon filter and degassed prior to use. The injection volume was20 µL. The eluents were monitored using DAD at 225 nm and FL at $\lambda_{ex/em}$, 225/310 nm.

Preparation of *p*CPA

*p*CPA was prepared by alkaline hydrolysis of MFX using 2 M NaOH following the reported method (El-Bardicy *et al.*, 2007 a).

Preparation of solutions

Preparation of stock and working standard solutions

Stock solution of 1000 µgmL^{-1} of MFX in 0.1 M HCl was prepared and kept in the refrigerator. Working standard solutions were prepared by suitable dilution with the mobile phase to cover the concentration range of 0.5-100 µg mL^{-1} and 0.05-20 µg mL^{-1} for DAD and FL, respectively. The stock solution should be freshly prepared daily.

Stock solution of 1000 µg mL^{-1} of *p*CPA was prepared by dissolving *p*CPA powder in 2 mL of methanol followed by dilution with distilled water. Working standard solutions were prepared by suitable dilution with the mobile phase to cover the concentration range of 0.25-100 µg mL^{-1} and 0.01-8 µg mL^{-1} for DAD and FL, respectively.

Preparation of dosage form solutions

Preparation of MFX tablets solution

A total of 20 Lucidril ®tablets were weighed and finely powdered. An accurate weight of the finely powdered sample equivalent to 50 mg of MFX was extracted into about 2 mL methanol with the aid of sonication for 1 min, using ultrasonic bath, then 20 mL of 0.01 M HCl were added and the sonication was continued for further 10 min. The mixture was filtered into a 50-ml volumetric flask. The residue was washed with two 10-ml portions of 0.01 M HCl and washings were added to the filtrate and diluted to volume with 0.01 M HCl. The concentration of this prepared assay solution is 1000 µg mL^{-1}. Further suitable dilutions were made with the mobile phase before chromatographic analysis.

Preparation of MFX vials sample solution

Fifty mg from Lucifort® vial was transferred into 50-mL volumetric flask containing about 2 mL of methanol. The powder was dissolved and the volume was completed with 0.01 M HCl to prepare 1000 µg mL^{-1}assay vials solution. Further suitable dilutions were made with the mobile phase before chromate-graphic analysis.

Procedure for forced degradation study

Forced degradation of MFX was carried out under neutral/ acid/ base hydrolytic, oxidative and photolytic stress

conditions. MFX powder was dissolved in distilled water (for neutral hydrolysis, using 6h study time), 0.1 M HCl (for acid hydrolysis, using 6h study time), 0.1 M NaOH (for base hydrolysis, using 5 min study time), all kept at ambient temperature. After the degradation time specified was elapsed, a neutralization step of acidic and alkaline degraded solution was done. Chromatographic analysis of the degraded samples was carried out, taking into consideration a suitable dilution with the mobile phase, to get concentrations of 100 and 10 μgmL^{-1} MFX for the DAD and FL, respectively, as initial concentrations.

For the oxidative degradation, MFX drug powder was dissolved in 8% H_2O_2 (prepared by dilution of hydrogen peroxide 30 % with distilled water), and allowed for stress degradation for 24h at ambient temperature. Another experiment was performed using 10% $H_2O_2$for 6 h at 80°C. Chromatographic analysis of the degraded samples was carried out, taking into consideration a suitable dilution with the mobile phase, to get concentration of 500 μgmL^{-1} MFX for the DAD and FL, as initial concentration.

Photo-degradation was carried out on solid drug substance and on solutions of MFX prepared in 0.01 M HCl and in distilled water. The solid and the solutions were irradiated with UV radiation having peak intensity at 254 nm for 3 h. After the specified time, the mobile phase was used to reconstitute the powder and to suitably dilute the solutions in order to get concentrations of 100 and 10 $\mu g\ mL^{-1}$MFX for the DAD and FL, respectively as initial concentrations.

Kinetic and stability study of MFX

Into a set of volumetric flasks, separate accurate volumes of a freshly prepared stock standard solution of MFX, 1000 $\mu g\ mL^{-1}$ in distilled water, were diluted with a suitable volume of the medium to be investigated, to give final concentrations of 100 and 20 μgmL^{-1}MFX for the DAD and FL, respectively. The kinetic studies were performed at ambient temperature. The concentration of the remaining MFX was monitored at suitable time intervals, taking into consideration that the analysed solution was double diluted with the mobile phase before chromatographic analysis.

In vitro dissolution study of MFX tablets

In vitro dissolution testing of Lucidril ® tablets was performed with USP apparatus 2 (paddle method) using dissolution medium, 900 mL of HCl of pH 1.2 as simulated gastric medium. A paddle stirrer was used for 60 min at a stirring rate of 100 rpm. The temperature of the cell was maintained at 37 ± 0.5°C.

Five mL sample aliquots were withdrawn at time intervals of 5 min, and immediately replaced with equal volume of the fresh dissolution medium to maintain constant total volume. These aliquots were filtered with 0.45 µm millipore filter and diluted 20 folds and 200 folds for DAD and FL, respectively, with the mobile phase before chromatographic analysis. The drug dissolved was calculated from chromatographic analysis data with reference to a standard MFX solution analysed in parallel. Then, the cumulative percentages of the dissolved drug were calculated. The dissolution data were obtained by averaging three parallel studies.

RESULTS AND DISCUSSION

Method development and optimization of chromatographic conditions

In developing and optimization of the HPLC method, 3 objectives were considered; first, establishing a stability indicating method for the analysis of MFX and *p*CPA, with best resolution from oxidative degradation products, second, setting conditions which favor the stability of MFX as it is highly susceptible to hydrolysis, third, maximizing the sensitivity, especially speaking, fluorescence detection.

The first study in the method development was concerned with the chromatographic separation of MFX and *p*CPA, using two tested columns; Zorbax SB-C8 (250 x 4.6 mm) and Zorbax Eclipse SB-C18 (250 x 4.6 mm);and a mobile phase consisting of 20mM acetate buffer adjusted to pH 3 (using acetic acid) : methanol (65: 35, v/v). The results referred to un-significant difference, regarding retention, resolution and peak symmetry. The C18 column was selected for the following studies. The second study aimed to compare between 20mM phosphate buffer of pH 3 and 20mM acetate buffer of pH 3, both at a ratio of buffer: methanol (65: 35, v/v), The results referred to un-significant difference, regarding retention, resolution, peak symmetry and UV detection sensitivity. However, phosphate buffer showed fluorescence intensity (peak area) nearly double that of acetate buffer, so phosphate buffer was chosen as optimum for the following studies. The next study dealt with the comparison between methanol and acetonitrile as an organic modifier, both at a ratio of 20mM phosphate buffer of pH 3: organic modifier (65: 35, v/v). About 35 % enhancement in FL detection was observed with acetonitrile with no noticeable difference in chromatographic performance and in UV detection sensitivity. Accordingly, acetonitrile was selected as the optimum organic modifier. Other study was performed using 20mM phosphate buffer of pH 3, with variation of acetonitrile ratio from 25% to 60%. The results showed that at acetonitrile % > 40%, shorten analysis time with poor resolution was obtained while acetonitrile % ≤ 30% resulted in marked retardation in chromatographic separation. Accordingly, acetonitrile % between 35% and 40% seems to be the best. Although 40% acetonitrile showed slight enhancement in FL detection, relative to 35%acetonitrile, the latter was chosen as optimum as it allows better resolution of MFX and *p*CPA from oxidative degradation products.

The final study, in optimizing chromatographic conditions, targeted pH. Two factors were considered, the pH-dependent stability of MFX and the pK_a of *p*CPA (3.56). The pH limit for best stability of MFX could be deduced from reported first order rate constant, k (min^{-1}) at pH 4 (1.19×10^{-4}) and pH 5 (2.39×10^{-4}), both correspond to $t_{90\%}$ 15h and 6.6h, respectively(Cooke *et al.*, 1979; Ohta *et al.*, 1986). So, working in pH ranged from 2 to 5 is practically possible, however, at pH 2,

*p*CPA showed significant retarded elution, also at pH ≥ 4, it directed towards too early elution (ionized species predominate), resulting in poor resolution from MFX peak. Accordingly, pH 3 was chosen as a working pH. It was advantageous that the isocratic elution achieved good chromatographic performance and analysis time less than 20 min for separation of MFX from all possible degradation products. DAD was set at 225 nm to allow for best method sensitivity. Both MFX and *p*CPA show strictly overlapping UV spectra showing maximum absorption at 225 nm.

To our knowledge, no reported article has studied the native fluorescence properties of MFX to establish an analytical method. *p*CPA is the hydrolytic product, also the active metabolite, of MFX(Cooke *et al.*, 1979). Its structure is closely related to chlorophenoxyacetic acid herbicides. Photodegraed-induced fluorimetric methods for the determination of chlorophenoxyacetic acid herbicides have been developed (Eremin *et al.*, 1996; Garcia *et al.*, 1996; Garcia-Campana *et al.*, 2001; Almansa Lopez *et al.*, 2003).The authors have claimed that all chlorophenoxyacetic acid herbicides have no native fluorescence. However, Schussler (1990) has developed an HPLC-fluorescence detection method based on measurement of native fluorescence of chlorophenoxy derivatives, without any derivatization, in water down to ng levels. He showed that4-chloro-2-methylphenoxyacetic acid and 4-chloro-2-methylphenoxybutanoic acid are natively fluorescent species, while 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenoxybutanoic acid are not fluorescent. The present work targeted the native fluorescence of MFX and *p*CPA to establish a new analytical HPLC-FL method. The FL detector was set at $\lambda_{ex/em}$, 225/310 nm, to achieve optimum sensitivity.

Figure 1 shows the typical chromatograms for the separation of MFX and *p*CPA mixture at retention times 3.81 ± 0.06 and 9.82 ± 0.08 min, respectively using the proposed HPLC-DAD-FL method.

System Suitability Parameters were assessed to ensure adequate performance of the chromatographic system. Retention time (R_t), capacity factor (k′), number of theoretical plates (N), peak resolution (R_s), selectivity (α) and tailing factor (T) were evaluated for six replicate injections of MFX and *p*CPA standard solutions at concentrations of 20 and 8 µg mL^{-1}, respectively. The results presented in Table1 are within the acceptable limits.

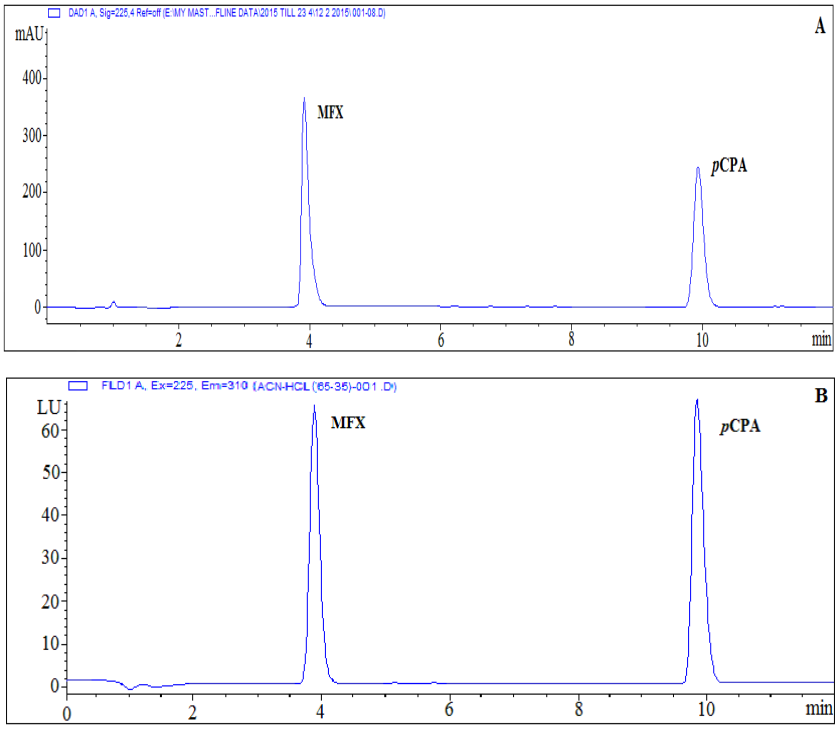

Fig. 1: HPLC chromatogram of 20- µL injection of (A) a mixture containing 40µg mL^{-1} MFX and 15µg mL^{-1}*p*CPA using DAD at 225 nm, (B) a mixture containing 5µg mL^{-1} MFX and 1µg mL^{-1}*p*CPA using FL at $\lambda_{ex/em}$, 225/310nm.

Table 1: System suitability parameters[a] of the developed HPLC-DAD-FL method.

Parameter	MFX	pCPA
Retention time (min) Rt±SD (n=6)	3.81±0.06	9.82±0.08
Capacity factor (k′)	2.81	8.82
Tailing factor (T)	1.11	1.13
Number of theoretical plates (N)	5806	16143
Selectivity (α)	3.14	
Resolution (Rs)	16.61	

[a] system suitability parameters were evaluated for six replicate injections of MFX and *p*CPA standard solutions at concentrations of 20 and 8 µg mL^{-1}, respectively, calculated as average of DAD and FL data.

Results from forced degradation studies

The study aimed to evaluate the specificity of the method towards the separation of MFX from the potential degradation products. The hydrolytic degradation of MFX (in neutral, acidic and alkaline solutions) is well known and is a matter of many researches (Cooke *et al.*, 1979; Tatsuhara and Tabuchi, 1980; Yoshioka *et al.*, 1982; Yoshioka *et al.*, 1983; Ohta *et al.*, 1986; Araman *et al.*, 1992; Otsuka *et al.*, 1994; El-Bardicy *et al.*, 2007 a; El-Bardicy *et al.*, 2007b; El-Nashar *et al.*, 2012). Table2 demonstrates the % remaining of MFX, it is clear that the reaction is base-catalysed. The chromatograms (Figures 2- 4) show good resolution of MFX from the hydrolytic product, *p*CPA. It should be noted that, N,N-dimethylamino ethanol is not detected as it is a non UV absorbing species.

Forced oxidative degradation was performed with 8% H_2O_2 for 24 h at ambient temperature and with 10% H_2O_2 for 6h at 80°C. The DAD and FL chromatograms of the stressed samples showed peaks for MFX, *p*CPA and three oxidation products I, II, and III at 12.2, 14.60 and 15.80 min, respectively, with good resolution of MFX and *p*CPA from the oxidation products (Figure 5). Analogus experiment was done on *p*CPA, HPLC analysis showed the same oxidation products I, II, and III at the same retention times (Figure 6). The stress oxidation reaction of MFX and *p*CPA can be explained in the view of chromatographic results. MFX is highly liable to hydrolysis, even in H_2O_2 aqueous solution, which most probably occurs in preference to oxidation. The readiness of MFX to get oxidized is slow. Identical oxidation products in the chromatograms of MFX and *p*CPA (as proved by retention times and UV spectra obtained by DAD) agreed with our assumption that these are the products of oxidation of *p*CPA. This means that hydrolysis of MFX occurs first then followed by oxidation of the hydrolytic product. Only small fraction of *p*CPA gets oxidized, even with 10% H_2O_2 and heating for 6h at 80°C.This is in line with the reported oxidative degradation reaction of *p*CPA by H_2O_2 which has been catalized photochemically to force the reaction to completion (Lang and Lunák, 2002). The oxidation products are natively fluorescent species, detected in the FL chromatogram.

HPLC chromatograms of photo-degraded MFX (UV irradiated solid or solutions) showed only *p*CPA peak, with no photo-degradation products. The hydrolysis of MFX was monitored in the % remaining of MFX (Table 2).

In all stress degradation studies, the peak purity data for MFX and *p*CPA were assessed (Table 2).

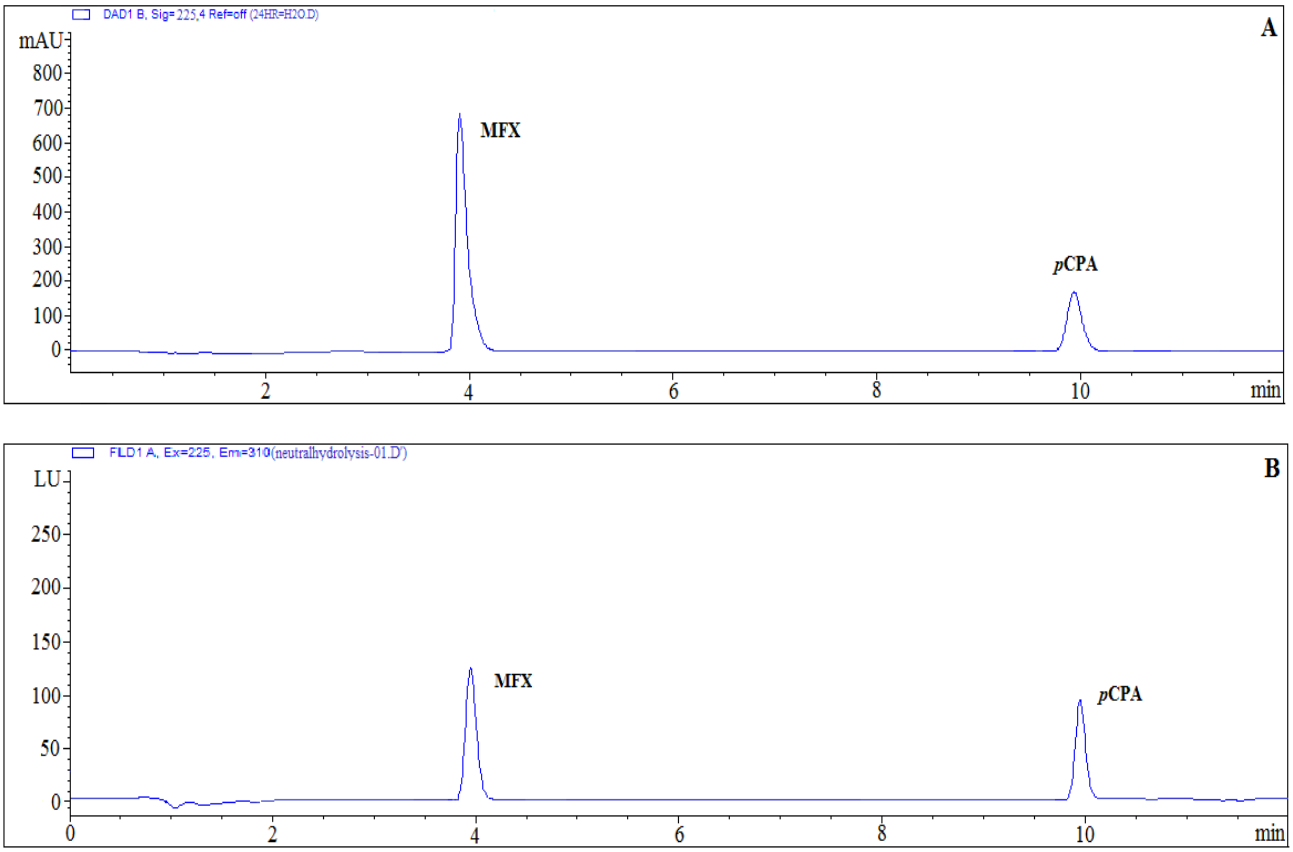

Fig. 2: HPLC Chromatogram of MFX after exposure to neutral hydrolysis for 6 hr at ambient temperature (A) using DAD at 225 nm (100 µg mL^{-1}, initial concentration), (B) using FLat $\lambda_{ex/em}$, 225/310 nm (10 µg mL^{-1}, initial concentration).

Table 2: Forced degradation studies.

Stress conditions	%remaining MFX		DAD Purity factor MFX	DAD purity factor *p*CPA
	DAD	FL		
Neutral hydrolysis, Distilled water, 6h, ambient temperature	83.3	84.1	999.983	999.991
Acid hydrolysis, 0.1 M HCl, 6h, ambient temperature	98.5	98.7	999.990	999.985
Base hydrolysis, 0.1 M NaOH, 5 min, ambient temperature	49.6	49.0	999.987	999.998
Oxidative degradation, 8% H_2O_2, 24h, ambient temperature	51.3	52.4	999.992	1000
Oxidative degradation, 10% H_2O_2, 6h, 80°C	40.3	39.5	999.995	999.981
Photo-degradation, Solid drug, at 254 nm, 3h	99.6	99.4	999.984	999.997
Photo-degradation, Solution in 0.01 M HCl, at 254 nm, 3h	98.7	98.6	999.989	999.983
Photo-degradation, Solution in distilled water, at 254 nm, 3h	90.6	91.1	999.997	999.992

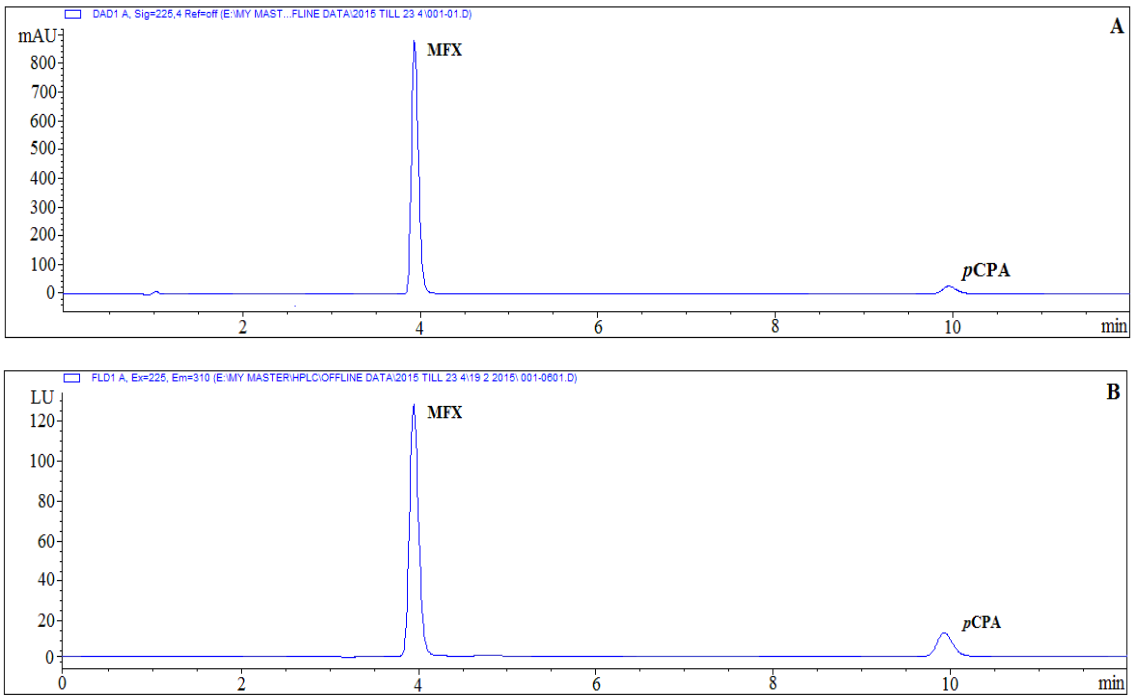

Fig. 3: HPLC Chromatogram of MFX after exposure to acid hydrolysis with 0.1 M HCl for 6 hr at ambient temperature (A) using DAD at 225 nm (100 µg mL $^{-1}$, initial concentration), (B) using FLat $\lambda_{ex/em}$, 225/310 nm (10 µg mL $^{-1}$, initial concentration).

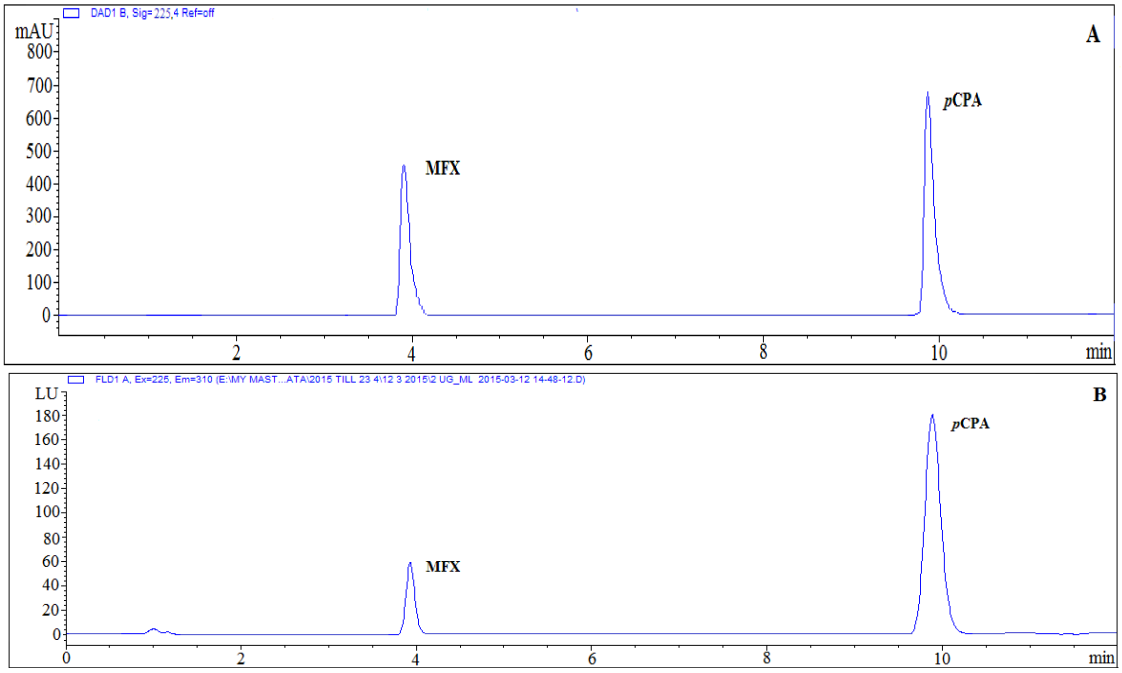

Fig. 4: HPLC Chromatogram of MFX after exposure to base hydrolysis with 0.1 M NaOH for 5 min at ambient temperature (A) using DAD at 225 nm (100 µg

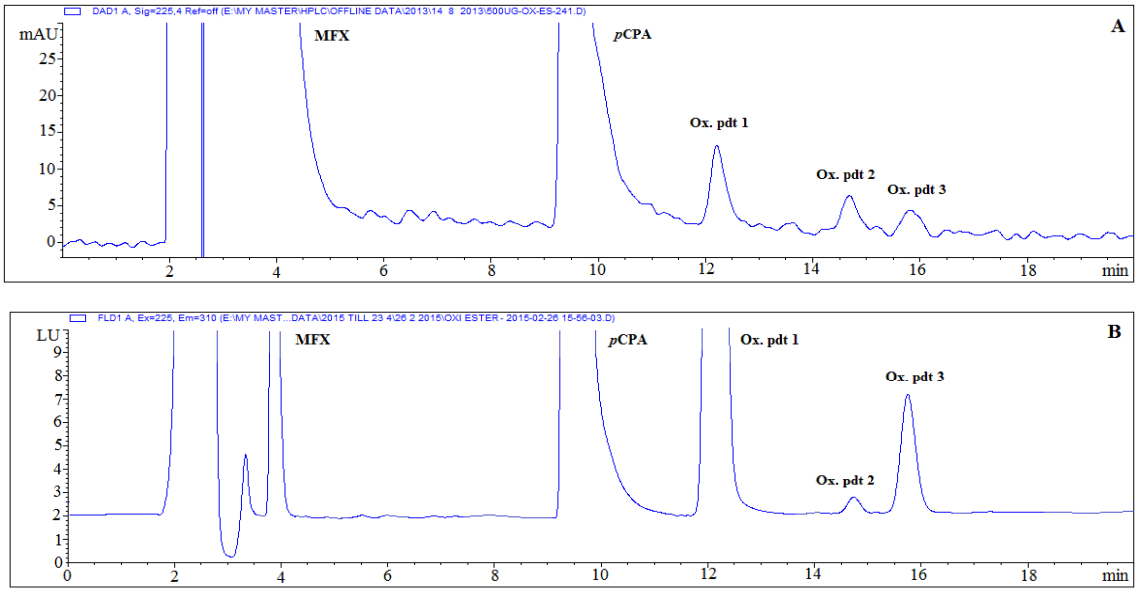

Fig. 5: HPLC chromatogram of 500 µg ml^{-1} MFX after exposure to oxidative degradation with 8 % H_2O_2 for 24 h at ambient temperature (A) using DAD at 225 nm , (B) using FL at $\lambda_{ex/em}$, 225/310 nm.

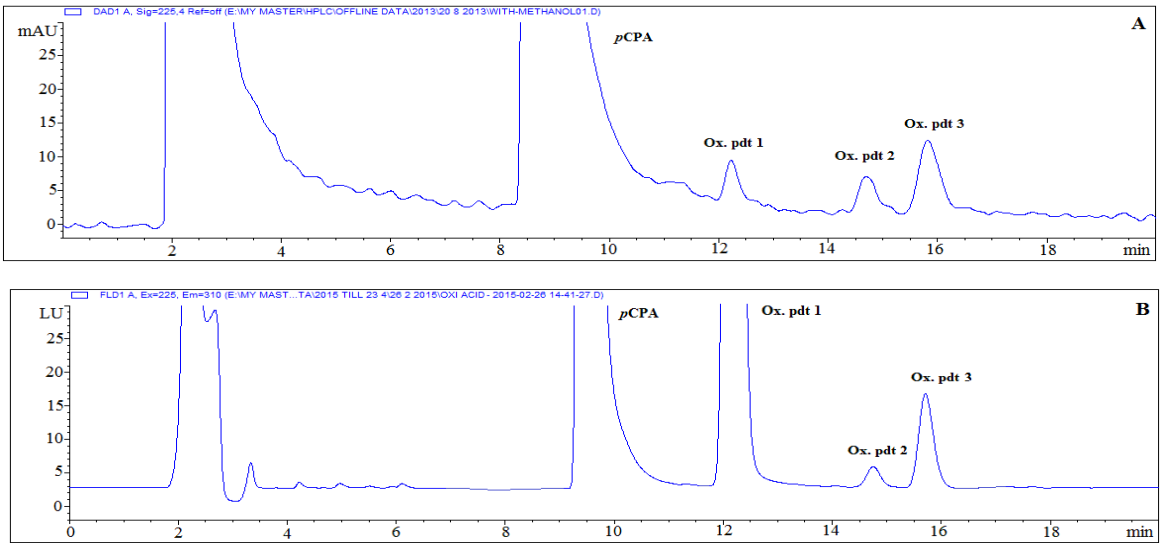

Fig. 6: HPLC chromatogram of 500 µgml^{-1}*p*CPA after exposure to oxidative degradation with 8 % H_2O_2 for 24 h at ambient temperature (A) using DAD at 225 nm, (B) using FL at $\lambda_{ex/em}$, 225/310 nm.

Table 3: Analytical parameters for the determination of MFX and *p*CPA using the proposed HPLC-DAD-FL method.

	DAD		FL	
Parameters	**MFX**	***p*CPA**	**MFX**	***p*CPA**
Wavelength (nm)	225	225	$\lambda_{ex/em}$ =225/310	$\lambda_{ex/em}$ =225/310
Linearity range (µg mL^{-1})	0.5-100	0.25-100	0.05-20	0.01-8
Intercept (a)	-1.34	6.74	-2.58	3.66
S_a[a]	2.09	6.51	3.28	4.20
Slope (b)	50.01	96.10	62.64	321.82
S_b[b]	0.05	0.73	0.37	1.30
RSD% of the slope (S_b%)	0.10	0.76	0.59	0.40
Correlation coefficient (r)	0.99999	0.99990	0.99991	0.99994
$S_{y/x}$[c]	4.09	12.08	8.15	10.33
LOD[d] (µg mL^{-1})	0.05	0.04	0.005	0.001
LOQ[e] (µg mL^{-1})	0.16	0.13	0.018	0.005

[a]S_a,Standard deviation of the intercept. [b]S_b, Standard deviation of the slope. [c]$S_{y/x}$, Standard deviation of residuals (standard error of estimates) . [d]Limit of detection. [e]Limit of quantitation.

Validation of the proposed method

Linearity and concentration ranges

Standard solutions of six concentration levels, for MFX and *p*CPA, were prepared and injected twice for each level onto the HPLC system. Table 3 presents the linearity data and statistical parameters including concentration ranges, slope (b) and intercept (a) of the linear regression equations, correlation coefficients and standard deviations of the intercept (S_a), slope (S_b) and residuals ($S_{y/x}$). Regression analysis for the calibration curves showed good linear relationship over the concentration ranges stated in the table as judged by the correlation coefficient values (r > 0.9999)and RSD% of the slope (S_b%) which did not exceed 0.76%.

Limits of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated in accordance to the USP and ICH guidelines based on signal-to-noise ratio values(ICH, 2005). Data given in Table 3 reveals that the proposed method is sensitive enough to detect concentrations down to 0.05 and 0.04 µg mL^{-1} (DAD); and 0.005 and 0.001µg mL^{-1} (FL) for MFX and *p*CPA, respectively.

Accuracy

Accuracy of the developed methods was determined by performing recovery experiments. Known amounts of standards MFX and *p*CPA at three concentration levels were fortified to pre-analysed degraded samples (MFX sample at 20 µg mL^{-1} concentration level was exposed to oxidative degradation using 10% H_2O_2 at 80°C for 6 h, pre-analysed results were 8.05µg mL^{-1} MFX and 5.06 µg mL^{-1}*p*CPA). Three replicate samples of each concentration level were prepared and the percentage recovery at each level was determined (Table4), taking into consideration that the peak area of the standard added was calculated by the difference in peak area between fortified and unfortified samples. The mean percentage recoveries obtained ranged from 98.2 to 101.6%. The percentage relative standard deviation (RSD%) did not exceed 1.5 %.

Table 4: Accuracy of the proposed HPLC-DAD-FL method.

Amount of standard MFX fortified[a] µg mL^{-1}	Amount of standard *p*CPA fortified[a] µg mL^{-1}	Mean % recovery[b]± SD[c] of fortified MFX	*p*CPA
DAD			
1	1	98.3±0.62	101.0±0.91
20	20	101.6±0.89	100.4±0.78
40	40	99.6±1.10	98.9±1.3
FL			
0.5	0.02	100.7±0.59	99.2±0.81
5	1.0	99.9±1.42	100.9±0.97
10	2.0	101.3±0.75	98.2±1.50

[a] standards fortified to pre-analysed degraded samples containing 8.05 µg mL^{-1} MFX and 5.06 µg mL^{-1}*p*CPA. [b] recovery obtained by difference in peak area between fortified and unfortified pre-analysed degraded samples. [c] standard deviation (n=3).

Precision

Intra-day and inter-day precision were evaluated by analyzing three replicates over three concentration levels of standards MFX and *p*CPA, within the same day (intra-day repeatability) and on three consecutive days (inter-day precision). The intra-day and inter-day precision (% RSD) was found to be less than 2% (Table5), indicating that the method is precise.

Table 5: Intra-day and inter-day precision of the proposed HPLC-DAD-FL method.

Analyte	Conc µg mL^{-1}	Intra-day RSD%[a]	Inter-day RSD%[a]
DAD			
MFX	0.5	0.61	0.98
	40	1.23	1.47
	100	0.90	1.75
***p*CPA**	0.25	0.76	0.90
	40	0.81	0.99
	100	1.01	1.23
FL			
MFX	0.05	1.12	1.34
	10	0.79	0.82
	20	0.63	0.93
***p*CPA**	0.01	0.96	1.45
	4	1.58	1.76
	8	0.84	1.19

[a] relative standard deviation % (n=3)

Specificity

The specificity of the developed HPLC-DAD-FL method was demonstrated by its ability to measure accurately and specifically MFX and *p*CPA in the presence of other potential degradants or excipients (ICH, 2005).

Photodiode array detector was used to evaluate the homogeneity and purity of the chromatographic peaks. Chromatographic peak purity data was obtained from the Agilent Chemstation Software (Stahl, 2003).A similarity factor of 0 indicates no match and that of 1000 indicates identical spectra. Generally, values very close to the ideal similarity factor (greater than 995) indicate that the spectra are very similar (Stahl, 2003).The similarity factors for, MFX and *p*CPA peaks in the chromatograms of stressed samples, assayed tablets and vials were found to be in the range of 999.983- 1000. All these factors lie within the corresponding automatically computed noise threshold limits indicating pure peaks. The threshold limits were found to be in the range of 999.936- 999.974.

The specificity of the HPLC methods is also illustrated from chromatograms obtained with the mixture solution of standard MFX/*p*CPA, stressed samples, tablets solution and vials solution. Well-resolved peaks were observed for MFX and *p*CPA in all the studied chromatograms(Rs =16.61), and for *p*CPA and oxidation product I in the stressed samples chromatograms (Rs =3.85). Additionally, either of MFX or *p*CPA showed identical retention times in all the studied chromatograms. No interfering peaks from any of the excipients appear in tablets chromatogram.

Method specificity was further confirmed by the recovery experiments carried out on standards MFX and *p*CPA added to degraded samples (Table 4). This indicates that the

Table 6: Assay results of MFX and *p*CPA in commercial pharmaceutical preparations using the proposed HPLC-DAD-FL method.

Batch number	Found MFX[a]				Found*p*CPA[a]			
	DAD		FL		DAD		FL	
	Mean%	RSD%	Mean%	RSD%	Mean mg/tablet	RSD%	Mean mg /tablet	RSD%
Lucidril® tablets								
CEE1662	96.5	0.96	96.6	0.76	5.69	0.85	5.53	0.97
EEE1518	96.6	0.72	96.4	1.20	5.54	0.79	5.85	0.60
DAE0153	95.9	1.18	96.0	0.67	6.66	1.16	6.50	1.00
Lucifort® vials								
DDE1158	96.7	0.72	96.8	0.73	10.73	0.84	10.40	1.16
DAE0057	96.6	0.83	96.6	0.77	11.05	0.83	11.05	0.87
CDE1089	96.4	0.79	96.3	0.62	11.70	0.99	12.03	0.78

[a]mean of five replicates.

developed method was specific and can be applied to simultaneous estimation of MFX and *p*CPA in kinetic stability study of MFX and in analysis of pharmaceutical formulations, to monitor the % degraded.

Stability of Solutions

Standard solution of MFX, prepared in 0.1 M HCl, is stable at ambient temperature for up to8h only (regarding the $t_{98\%}$, time required for the drug concentration to fall to 98% of the original value), so it should be freshly prepared daily. Standard solution of *p*CPA prepared in methanol/water is stable and can be kept in refrigerator for about one week. The stability was verified by chromatographic analysis and monitoring the respective retention time and peak area.

Robustness

To evaluate the robustness of the developed HPLC-DAD-FL method, small deliberate variations in optimized methods parameters were made once at a time (ICH, 2005). The parameters included variation of flow rate (± 0.05 mL min^{-1}),detection wavelengths (± 1 nm), pH of the phosphate buffer (± 0.05), and % of acetonitrile in the mobile phase (± 0.5%). Replicate injections (n=3) of standard solutions (40 µg mL^{-1}MFX and 15µg mL^{-1}*p*CPA for DAD) (5µg mL^{-1}MFX and 1 µg mL^{-1}*p*CPA for FL) were used. These variations have no significant effect on the peak area, retention time, tailing factor and resolution of the analytes.

Applications of the developed method

Assay of MFX Tablet and Vial dosage forms

The developed HPLC-DAD-FL method was applied to the assay of MFXcommercial pharmaceutical formulations (Lucidril® tablets and Lucifort® vials).Both the intact drug and its hydrolytic degradation product, *p*CPA were determined in 3 different batches. It should be noted that the samples were extracted with methanol prior to 0.01 M HCL to dissolve any present *p*CPA. The stability of MFX in methanol/ HCl was proved in the current kinetic study.In all batches, the mean % of MFX found was in the range of 95.9 – 96.8% and for *p*CPA, the mean amount found was in the range of 5.53 – 6.66 mg per tablet and 10.40 – 12.03 mg per vial (Table 6). The hydrolysis of MFX in commercial dosage forms is attributed to heat and humidity of storage conditions. The results agreed with the reported study on the hydrolysis of MFX in commercial tablets (Yoshioka *et al.,* 1983).

Kinetic stability studies of MFX

Hydrolysis of MFX, in aqueous solution, is known to be first order reaction and pH dependent (Ohta *et al.,* 1986). Some kinetic studies on MFX stability have been reported. These include MFX stability in solid dosage form to investigate effect of temperature and humidity (Yoshioka *et al.,* 1982; Yoshioka *et al.,* 1983), hydrolysis of MFX in buffer solutions at pH 4 to 11 (Cooke *et al.,* 1979)and degradation reaction of MFX in 1M, 1.5M and 2M NaOH (El-Bardicy *et al.,* 2007 a).

In the present work, the kinetic studies on MFX stability aimed to serve drug analysis, in view of selecting the most appropriate solvents to prepare stable MFX solutions or even optimization of the analytical method conditions (like pH of the solution, % organic solvent) to compromise drug stability and method sensitivity. So, the current kinetic study included stability of MFX in aqueous solutions of different pH values and in aqueous/organic solvent mixtures, as summarized in Table 7. The developed HPLC-DAD-FL method was applied to monitor the percentage remaining of MFX in the investigated media. The kinetic parameters were calculated using equations (1) and (2) (Sinko, 2006):

$$\log C_t/C_0 = -k / 2.303\, t \quad (1)$$

$$t_{1/2} = 0.693/k \quad (2)$$

where, k is the first order reaction rate constant, C_0 is the concentration of MFX at time t = 0 and C_t is its concentration at time t. The degradation reaction rate constant was calculated from the slope of regression line representing the correlation of log % remaining of MFX and time. The kinetic parameters, presented in Table 7, include: k (rate constant), $t_{1/2}$ (half-life time) and $t_{90\%}$ (time required for the drug concentration to fall to 90% of the original value).

From the data given in Table 7, our findings could be reviewed in the following remarks: first, MFX is not stable enough

Table 7: Kinetic parameters of MFX in different media using the proposed HPLC-DAD-FL method.

Media	K (min^{-1}) [a]		t (min) [b]			
			$t_{1/2}$		$t_{90\%}$	
	DAD	FL	DAD	FL	DAD	FL
0.1 M NaOH	$14x10^{-2}$	$15x10^{-2}$	4.95	4.62	0.75	0.70
Ammonium carbonate: acetonitrile (70: 30) pH 8.6	$96x10^{-3}$	$99x10^{-3}$	7.2	7.0	1.1	1.1
Methanol: phosphate buffer pH 7.4 (1:1)	$61x10^{-3}$	$62x10^{-3}$	11.4	11.2	1.7	1.7
20mM phosphate buffer pH 7.4	$48x10^{-3}$	$44x10^{-3}$	14.4	15.75	2.2	2.4
Buffer for reconstitution of vial[c]	$19x10^{-3}$	$18x10^{-3}$	36.5	38.5	5.5	5.8
Methanol: water (1:1)	$59x10^{-4}$	$55x10^{-4}$	117.5	126.0	17.8	19.1
Methanol	$33x10^{-4}$	$35x10^{-4}$	210.0	198.0	31.8	30.0
Distilled water	$51x10^{-5}$	$48x10^{-5}$	1358.8	1443.8	206.6	218.8
Methanol: HCl 0.1M (1:1)	$21x10^{-5}$	$20x10^{-5}$	3300	3465.0	500	526.9
Methanol : 20 mM acetate buffer pH 3 (1:1, v/v)	$17x10^{-5}$	$19x10^{-5}$	4076.5	3647.4	617.6	554.6
Normal saline	$17x10^{-5}$	$18x10^{-5}$	4076.5	3850.0	617.6	585.4
20 mM acetate buffer pH 3	$73x10^{-6}$	$70x10^{-6}$	9493.2	9900.0	1438.4	1505.4
Acetonitrile : 20mM phosphate buffer pH 3 (35: 65, v/v)	$71x10^{-6}$	$69x10^{-6}$	9760.6	10043.5	1484.2	1527.2
0.1 M HCl	$42x10^{-6}$	$40x10^{-6}$	16500	17325	2509.0	2634.5
0.01 M H_2SO_4	$35x10^{-6}$	$36x10^{-6}$	19800	19250	3010.9	2927.2

[a] rate constant.
[b] t, time required for MFX to fall to 50% ($t_{1/2}$) and 90% ($t_{90\%}$) of the original value.
[c] composed of 18.06 mg disodium phosphate and 17.61 mg potassium dihydrogen o-phosphate ,pH 6.82.

Table 8: Dissolution study of Lucidril® tablets using the proposed HPLC-DAD-FL method.

Time (min)	Average MFX dissolved[a]			
	DAD		FL	
	mg	%	mg	%
5	43.3	17.3	44.0	17.6
10	106.0	42.4	108.0	43.2
15	137.0	54.8	134.0	53.6
20	191.5	76.5	194.0	77.6
25	213.3	85.3	215.8	86.3
30	218.5	87.4	219.3	87.7
40	224.8	89.9	226.5	90.6
50	229.3	91.7	231.0	92.4
60	233.5	93.4	234.5	93.8

[a] The dissolution data were obtained by averaging three parallel studies.

in water, its $t_{90\%} \approx 210$ min, alsoMFX in methanol, its $t_{90\%} \approx 31$ min, so either water or methanol is not suitable for preparation of standard MFX solution. Second, $t_{1/2}$ of MFX in ammonium carbonate: acetonitrile (70: 30) of pH 8.6 ≈ 7 min, so it is not suitable to be used as mobile phase.Third, $t_{1/2}$ of MFX in 0.1M NaOH is < 5 min; this is in agreement with that in reference (Cooke *et al.,* 1979) and it seems to be more realistic than the kinetic data given for MFX hydrolysis in NaOH, in reference (El-Bardicy *et al.,* 2007 a).

As a conclusion, some points, seem to be valuable and should be high-lighten. First, MFX exhibits best stability in aqueous acidic solution of pH 1-3 (HCl, H_2SO_4 and acetate buffer). Second, methanol fastens the degradation reaction, as seen in mixtures of methanol with water, HCl, and buffers of pH 3 and 7.4. However, acetonitrile and methanol added to acidic aqueous solutions, still keep good stability required for performing analytical experiments.

In vitro dissolution study of tablet dosage form

The developed HPLC-DAD-FL method was applied to calculate the amount of MFX released from Lucidril® tablets (Table 8). The results showed that more than 75% of the drug dissolved within 20 min and about 93% of the labeled MFX per tablet eventually dissolved in 60 min. The dissolution profile for tablets was considered satisfactory and agreed with the results reported for lucidril® tablets, manufactured by Minapharm, Egypt (El-Nashar *et al.,* 2012).

CONCLUSION

The present work described a simple, sensitive and reliable HPLC-DAD-FL method for the assay of MFX and *p*CPA. The paper is the first to exploit the native fluorescence properties of MFX and its hydrolytic product, *p*CPA, to establish a sensitive HPLC method. The limits of quantitation were found to be 18 and 5 $ngmL^{-1}$ for MFX and *p*CPA, respectively with fluorescence detection without any derivatization.

The developed method is superior to the other previously reported analytical methods, as the present work considered hydrolytic, oxidative and photolytic degradations. To our knowledge, the previous attempts have concerned only with hydrolytic degradation. The developed HPLC-DAD-FL method was successfully applied to the analysis of MFX tablets and vials, to determine the intact drug as well as the hydrolytic product, *p*CPA, also applied to the dissolution testing of MFX tablets. Further, the method was applied to kinetic studies on MFX hydrolytic degradation reaction. Kinetic parameters including degradation rate constant, $t_{1/2}$ and $t_{90\%}$ were estimated. The kinetic

data are especially valuable to give attention about the most appropriate media, which favor MFX stability, to be a guide in establishing analytical method conditions.

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How to cite this article:

Marwa Said Moneeb, Feda Elgammal, Suzy Mohamed Sabry., Development and validation of a stability indicating HPLC-diode array-fluorescence method for the determination of meclofenoxate hydrochloride and *p*-chlorophenoxyacetic acid. J App Pharm Sci, 2016; 6 (07): 001-011.