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Research Article

DETERMINATION OF GEMFIBROZIL AND FENOFIBRATE IN PHARMACEUTICALS IN PRESENCE OF THEIR DEGRADATION PRODUCTS

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ABSTRACT

The developed study portrays stability indicating methods for the determination of gemfibrozil and fenofibrate in pharmaceuticals. Three methods were presented for the determination of gemfibrozil in presence of its acid degradation products. The first method was based on HPLC separation of gemfibrozil from its degradation products on a reversed phase ODS column and UV detection at 276 nm. The second method was based on TLC separation followed by densitometric measurement of the spots. The third method was based on measuring the native fluorescence of the cited drug at $\lambda_{em.}$405 nm upon excitation at $\lambda_{ex.}$ 300nm. The three proposed methods were successfully applied to the determination of gemfibrozil in the range of 4-20 µgmL^{-1}, 4-20 µg/spot and 0.1-1.1 µgmL^{-1} for the HPLC, TLC and spectrofluorimetric methods, respectively.

Two methods were presented for the determination of fenofibrate in presence of its acidic and basic degradation products. The first method was based on TLC separation of the drug from its degradation products, followed by densitometric measurement of the spots at 286 nm. The second method was based on measuring the peak amplitude of the first derivative curve at 248.4 nm for the drug in presence of its acidic degradates and at 250.8 in the presence of its basic degradate. The two proposed methods were applied to the determination of fenofibrate in the range of 1-9 µg/spot and 2-12 µgmL^{-1} for the TLC and the spectrophotometric methods, respectively.

Commercial tablets and Laboratory prepared mixtures for both drugs in different proportions were assayed using the developed methods; also the fluorimetric method was applied to spiked human plasma. The analytical results were quite good in all cases. The main degradation products were subjected to IR and Mass spectrometry to confirm their structures and scheme for their formation.

Keywords*:* Gemfibrozil, Fenofibrate, Degradation products, HPLC, TLC densitometric measurement, Spectrofluorimetric measurement, First derivative.

INTRODUCTION

Gemfibrozil (Gf) $C_{15}H_{22}O_3$; Pentanoic acid, 5-(2,5-dimethyl phenoxy)-2.2-dimethyl- 2,2 dimethyl-5-(2.5-xylyloxy) valeric acid, **Fenofibrate** (Ff) $C_{20}H_{21}ClO_4$; Isopropyl2-(4-(4-Chlorobenzoyl) phenoxy)-2-methyl propionate, Scheme (1 a & b). They are lipid regulating drugs; they activate a catabolic system for fatty acid in liver cells by interacting with nuclear receptor which regulates gene transcription then increase degradation of VLDL (very low density lipoproteins). They are described as antihyperlipidimic drugs. They are taken by mouth for the treatment of hyperlipidemia. Several methods have been reported for the determination of Gf in bulk powder, in biological fluids and in pharmaceutical formulations. They include: HPLC [1-6], Spectrofluorimetric [7, 8], I.R. [9, 10] and GC [11-12]. Gf is official in USP [13]. Also, several methods have been reported for the determination of Ff; they include: HPLC [14-18], GC [19], MS [20, 21], Densitometric TLC-Chromatographic [22-23], spectrophotometric[24] methods, Ff is official in USP [25]. While, the literature had several HPLC methods and few spectrophotometric and spectrofluorimetric methods yet, none of them determined Gf in presence of its degradates. Also, the reported methods had not mentioned any TLC-Densitometric methods for the determination of both Gf and Ff in presence of their degradates. Accordingly; the aim of the present study was to develop simple, rapid, and sensitive stability indicating HPLC, TLC-densitometric, Fluorimetric and first derivative spectrophotometric methods for the determination of the cited drugs.

MATERIALS AND METHODS

Instrumentation

- HPLC system consists of Agilant 1100 series control module by agilent chemstation for HPLC equipped with a quaternary pump injector with a 20ml loop and a U.V.-variable wavelength detector model agilent 1100 series. Column used was a nucleosil C18 RP column (250 x 4 mm, 10 µm particle size)

- Ultrasonic bath J.P. selecta (Barcelona , Spain).

- Shimadzu-dual wave length lamp flying spot CS 9301 PC software densitometer (Tokyo, Japan).

- U.V. short wave length lamp (254nm).

- Silicagel TLC plates 60 F_{254} on alumina card 20cmx10cm, 0.2mm thickness (Fluka, Buchs, Switzerland).

- Pre-coated TLC glass plates G-50 UV_{254} 20x20cm, 0.2mm Silica F_{254} (Machery-Nagel, Germany), Jars glass 20x22x10cm. 100ml syringe (Hamilton U.S.A).

- Shimadzu RF-1501 spectrofluorimeter (Japan).

- Hettachi centrifugator (Germany).

- Shimadzu U.V-VIS spectrophotometer with two matched 1 cm quartz cell.

- HANNA pH meter (EOS, Egypt).

Solvents and chemicals

All solvents and chemical used were of analytical grade.

- HPLC-grade acetonitril and methanol were purchased from LAB-SCAN Analytical science (Dublin-Ireland).

- Chromatographic grade methanol, hexane and tetrahydrofurane used were purchased from LAB-SCAN Analytical Science (Dublin, Ireland).

- Ammonium dihydrogen orthophosphate was purchased from EL Nasr Pharmaceutical and Chemical Co.

- High pure water was prepared by double filtration using Millipore 0.45mm, White nylon HNWP47mm membrane filters.

- Ethylacetate, 33%NH3 from El Nasr Pharmaceutical and Chemical Co.

- Sodium hydroxide and hydrochloric acid from EL Nasr Pharmaceutical and Chemical Co.

- Spectroscopic grade methanol used for spectrophotometric method.

Samples

Pure samples*:* Gemfibrozil and Fenofibrate were kindly supplied by sigma Aldrich (Italy) their purity were labeled to be 99.95±0.92, 99.85±0.72 for Gf and Ff; respectively.

Market samples: Lopid tablets were packed by Pfizer Co. (Cairo, Egypt), B.N.9105 each tablet was claimed to contain 600 mg Gf.

Fenorate tablets were packed by Evapharm Co. (Cairo, Egypt), B.N. 5571 each tablet was claimed to contain 250 mg Ff.

Standard solutions

Gf stock solution: 1 $mgml^{-1}$ for TLC, 0.1$mgml^{-1}$ for HPLC, Fluorimetry and 1D in methanol.

Ff stock solution: 1 $mgml^{-1}$ for TLC, 0.1$mgml^{-1}$ for methanol in 1D.

Preparation of acidic degradation products of Gf

Accurately weighed 10mg were dissolved in 25 ml 7M HCl solution, refluxed at 90°C for 3 hours. During neutralization a precipitate was formed. The eluent was filtered. The filtrate was evaporated to dryness; the residue was dissolved in 10ml methanol and used allover the paper as stock solution of Gf degradation products.

Preparation of acidic and basic degradation products of Ff

Accurately weighed 10mg were dissolved in 25 ml 7M HCl solution and 7M NaOH for acidic and basic degradation products; respectively, refluxed at 90°C for 7 hours. During neutralization a precipitate was formed. The eluent was filtered. The filtrate was evaporated to dryness; the residue was dissolved in 10ml methanol and used all over the paper as stock solution of Ff acidic and basic degradation products.

Chromatographic conditions

For HPLC

Column used was a nucleosil C18 RP column (250 x 4 mm, 10 µm particle size). Mobile phase: acetonitril: methanol: ammonium dihydrogen orthophosphate 0.005% PH =7 (6o:30:10 by volume), It was filtered through Millipore filter 0.45um, white nylon HNWP 47mm and was degassed before use. Flow rate 0.8ml/min. The elution was monitored at 276nm, the injection volume was 20µl.

For TLC

Precoated 20×10 cm TLC Aluminum sheet silica gel F254 plates. Ten microliters spots of each stock solution were spaced 1.5 cm apart from each other and were applied 2 cm from the bottom edge of the plate. The plates were placed in a chromatographic tank previously saturated with the mobile phase that consists of ethyl acetate: methanol: NH3 (17:7:1 by volume) and hexane: tetrahydrofurane (12:10 by volume) for (Gf) and (Ff); respectively. The plate was left to develop by ascending chromatography through a distance of 16 cm at ambient temperature and then dried at room temperature. The spots were detected under U.V. lamp (254nm) and were scanned under the following condition:-Wavelength: 276nm and 286 for (Gf) and (Ff); respectively. Photomode: reflection, Lane: auto, Zero set mode: at start, Scan mode: zigzag, Difference: off, Lambda: single and Trace: off.

Optimization of Factors affecting Fluorescence Intensity

Four factors affecting fluorescence intensity were studied: *solvents*; including methanol, N/10 meth. NaOH, N/10 NaOH, N/10 H2SO4, acetone, propanol, ethanol and acetonitrile were tried. *Excitation wavelength*; different excitation wavelengths were tested in order to increase sensitivity and selectivity of the method in the range of 250-400nm for $\lambda_{ex.}$ And 350-550 nm for $\lambda_{em.}$. *Time*; was measured using 10 minutes intervals until 120 minutes to choose the best time that gives maximum fluorescence intensity. *Surfactants*; the cationic surfactant (cetyl pyridinium chloride), non-ionic surfactants (Tween 80) and different concentrations of anionic surfactant (SLS) .

Procedures

1-Construction of calibration curves

HPLC method

Aliquots of stock solution equivalent to 0.04-0.20mg of Gf were transferred into a series of 10 ml volumetric flasks and completed to volume with mobile phase to prepare different concentrations (4-20 µg ml^{-1}). Twenty microliters of each solution were injected then chromatographed using the previously mentioned specified chromatographic conditions. Peak areas were plotted versus concentrations, calibration curve was constructed and regression equation was computed.

TLC-densitometric method

Aliquots of stock solution equivalent to 4-20mg, 1-9mg for each of Gf and Ff, respectively were transferred into a series of 10 ml volumetric flasks and completed to volume with methanol. Ten microliter of each solution was applied using 10µl automatic pipette to prepare different concentrations (4-20 µg/spot) and (1-9 µg/spot) for Gf and Gf; respectively. Spots were spaced 1.5cm apart from each other and 2cm from the bottom edge, chromatographed as mentioned before, Calibration curve was constructed for each drug by plotting areas under peaks versus drug concentration and regression equation was computed.

Fluorimetric method

Aliquots of stock solution equivalent to 1-11µg of Gf were transferred into a series of 10 ml volumetric flasks and were completed to volume with N/10 NaOH to prepare different concentrations (0.1-1.1 µg ml^{-1}). The native fluorescence intensity was measured at $\lambda_{em.}$ 405 nm and at $\lambda_{ex.}$ 300 nm after 100 minutes. Calibration curve was constructed relating fluorescence intensity to the corresponding concentration; regression equation was computed.

1D method

Aliquots of stock solutions equivalent to 0.02-0.12 mg of Ff were transferred into a series of 10 ml volumetric flasks and completed to volume with methanol to prepare different concentrations (2-12 µg ml^{-1}). The spectra of the prepared solutions were scanned and stored in the computer, the first derivation spectra was recorded , peak amplitudes of the first derivative spectra of Ff at 248.4 nm for acidic degradates and at 250.8nm for basic degradate were measured. Calibration curve was constructed relating peak amplitude to the corresponding concentration, regression equation was computed.

Assay of laboratory prepared mixtures

HPLC method

Accurately measured aliquots of stock solution equivalent to 0.04-0.16mg of Gf were transferred into a series of 10 ml volumetric flasks, aliquots equivalent to 0.16-0.04 mg of degradation product stock solution were added, the flasks were completed to volume with mobile phase, to prepare different mixtures of acid degradation products (20-80%). The mixtures were then chromatographed using the specified chromatographic conditions.

TLC-densitometric method

Accurately measured aliquots of stock solutions equivalent to 4-16 mg of Gf and 0.9-8.1 mg of Ff were transferred into a series of 10 ml volumetric flasks, aliquots equivalent to 16-4 mg and 8.1-0.9 mg of degradation products stock solution were added for Gf and Ff; respectively, the volumes were completed with methanol. Ten microliter of each solution was applied using 10µl automatic pipette to prepare concentrations of acid-degradation products of Gf (20-80%) and mixtures of acid and base-degradation products Ff (10-90%). The mixtures were analyzed using the specified chromatographic conditions.

Fluorimetric method

Accurately measured aliquots of stock solution equivalent to 1-9 µg of GF were transferred into a series of 10 ml volumetric flasks, aliquots equivalent to 9-1 µg of degradation products stock solution were added, the flasks were completed to volume with solvent, to prepare different mixtures of acid degradation products (10-90%). The mixtures were then continued as under construction of calibration curve using the specified conditions.

1D method

Accurately measured aliquots of stock solution equivalent to 0.108-0.072 mg, 0.108-0.030 mg of Ff were transferred to two series of 10 ml volumetric flasks, aliquots equivalent to 0.072-0.108 mg, 0.03-0.108 mg of degradation products stock solutions were added to prepare different mixtures of acid degradate (10-40%) and of basic degradate (10-75%). The volumes were completed with methanol; the prepared mixtures were continued as under construction of calibration curve.

Assay of tablets

A quantity of mixed contents of 10 Lopid tablets equivalent to 25mg of Gf was transferred into 25 ml volumetric flask, the flask was half-filled with methanol, was shaken automatically for 15 min completed to volume with the same solvent and filtered.

A quantity of mixed contents of 10 Fenorate tablets equivalent to 25 mg of Ff was transferred into 25 ml volumetric flask, the flask was half-filled with methanol, shaken automatically for 15 min completed to volume with the same solvent and filtered.

Analysis were done for HPLC, TLC, Fluorimetry, 1D methods as previously mentioned under construction of calibration curves.

Procedure of spiked human plasma

Aliquots of stock solution equivalent to 1-11µg of Gf, 1ml was transferred into a series of centrifugation tubes. One milliliter of plasma and 3 ml of acetonitrile were added to each tube successively, were sonicated for 5 min. then the tubes were centrifuged at 3000 rpm for 30 min. One milliliter of the supernatants was transferred separately into 10 ml volumetric flasks, evaporated to dryness and the residues dissolved each in 1 ml acetone, the volumes were completed with N/10 NaOH. The procedure under construction of calibration curve was applied. The nominal content of the drug was determined using the corresponding regression equation.

Method validation

Linearty

For HPLC: Aliquots of stock solution of Gf equivalent to 0.04-0.20 mg were transferred into a series of 10 ml volumetric flasks and completed to volume with mobile phase. The solutions were chromatographed using the specified chromatographic conditions peak areas were plotted versus concentrations and regression equation was computed.

For TLC: Aliquots of stock solutions of Gf and Ff equivalent to 4 -20 mg, 1-9 mg, respectively, were transferred into a series of 10 ml volumetric flasks and completed to volume with methanol were applied using the specified TLC- chromatographic conditions. Calibration curve for each drug was constructed by plotting areas under peaks versus drug concentration and regression equation was computed.

For Fluorimetry: Aliquots of stock solution of Gf equivalent to 1-11 µg were transferred into a series of 10 ml volumetric flasks and completed to volume with N/10 NaOH. The native fluorescence intensity was measured at λem.405nm and at λex.300nm. Calibration curve was constructed relating fluorescence intensity (F) to the corresponding concentration (C) and regression equation was computed.

For 1D: Aliquots of stock solution of Ff equivalent to 0.02-0.12 mg were transferred into a series of 10 ml volumetric flasks and completed to volume with methanol. The spectra of the prepared solutions were scanned and stored in the computer, the derivation spectra were recorded, peak amplitudes of the first derivative spectra of Ff at 248.4 nm and 250.8 nm were measured. Calibration curves relating peak amplitude to the corresponding concentration of Ff were constructed.

Precision

Precision was calculated for intraday and interday using three concentrations each of triplicate determination and RSD were calculated from the corresponding regression equation.

Accuracy

Different concentrations of pure samples of Gf and Ff each of triplicate determinations were calculated from the corresponding regression equation and the mean percentage recoveries were then calculated.

Selectivity

For HPLC: laboratory prepared mixtures were prepared by accurately transferred aliquots equivalent to 0.04-0.16 mg of Gf stock solution into a series of 10 ml volumetric flasks ,aliquots equivalent to 0.16-0.04 mg of degradation products stock solution of Gf solution were added, the flasks were completed to volume with mobile phase, to prepare different mixtures of degradation products (20-80%). The mixtures were then chromatographed using the specified chromatographic conditions, the concentration of Gf were determined by substituting in the regression equation.

For TLC: laboratory prepared mixtures were prepared by accurately transferred aliquots equivalent to 4-16 mg of Gf and 0.9-8.1 mg of Ff into a series of 10-ml volumetric flasks, aliquots equivalent to 16-4 mg and 8.1-0.9 mg of degradation products stock solutions of Gf and Ff; respectively were added, the flasks were completed to volume with methanol, to prepare different mixtures of acidic degradation products of Gf (20-80%), different mixtures of acidic degradation products and basic degradation product of Ff (10-90%). The mixtures were analyzed using the specified chromatographic conditions.

For Fluorimetry: laboratory prepared mixtures were prepared by accurately transferred aliquots equivalent to 1-9 µg of Gf stock solution into a series of 10 ml volumetric flasks ,aliquots equivalent to 9-1 µg of degradation products stock solution of Gf were added, the flasks were completed to volume with solvent, to prepare different mixtures of degradation products (10-90%). The native florescence intensity was measured at $\lambda_{em.}$ 405 nm and at $\lambda_{ex.}$ 300 nm. The concentrations and the recovery percentages were calculated.

For 1D: laboratory prepared mixtures were prepared by accurately transferred aliquots equivalent to 0.108-0.072 mg, 0.108-0.030 mg of Ff stock solution into a series of 10 ml volumetric flasks, aliquots equivalent to 0.072-0.108 mg, 0.03-0.108 mg of the degradation products stock solutions of Ff were added, the flasks were completed to volume with methanol, to prepare different mixtures of acidic degradation products (10-40%) Ff and Basic degradation products (10-75%) Ff were prepared the spectra were scanned. The concentrations and the recovery percentages were calculated.

RESULTS AND DISCUSSION

Drug impurities and degradation products are produced during synthesis of the bulk materials or improper storage of the drug which could be due to hydrolysis, photolysis or oxidation [3]. The identification of these unknown impurities is required by regulatory guidelines and their toxicity and/or safety may also need to be evaluated dependent upon the nature and amount of the identified species.

HPLC method

Several HPLC methods were used for the resolution of interference between drugs and their impurities [1, 26-28], drugs and their metabolites [2] and different drugs [29]. Since no method has been reported in the literature for the determination of Gf in presence of its acid hydrolysis degradation products, therefore; it was thought necessary to develop a validated stability indicating HPLC method for the determination of Gf in bulk material and pharmaceutical preparation. To optimize the assay parameters several mobile phases, flow rates, wave lengths and temperatures were tried. A satisfactory separation between Gf and its degradation was obtained with acetonitrile: methanol: 0.005% ammonium dihydrogen phosphate (60:30:10 by volume), at flow rate 0.8mlmin^{-1}, UV detection at 276nm and at 20°C. The selectivity of the HPLC method was illustrated in figure 1, where complete separation of Gf and its degradation product was noticed with sharp symmetric peaks, clear baseline separation. Retention times were 1.85 ±0.2 min. and 1.41 ± 0.2 min. for intact Gf and its degradation product, respectively. The

mean percentage recovery of pure sample was found to be 99.2±0.04% (n=5) using the following regression equation

A=9.625C+17.5 r= 0.9999

Where A is the peak area, C is the concentration in µg ml^{-1} and r is the correlation coefficient. To assess the accuracy of the results, different pure samples of the studied drugs were analyzed by the suggested method and the concentrations were calculated from the corresponding regression equation. The proposed HPLC method could be used as stability indicating method for the determination of Gf in laboratory prepared mixtures in presence of up to 80% of its acid degradation product with mean percentage recovery of 100.7±0.84 (table 1). Moreover; the accuracy of the proposed method was evaluated by applying the standard addition technique. The results obtained were compared with the official HPLC method, which is an HPLC method using 3.9-mm x 30-cm L1 column, mobile phase consists of methanol : water : glacial acetic acid (80:19:1 by volume), flow rate of 0.8 $mlmin^{-1}$ and UV detection at 276 nm [13]. The calculated t & F values were less than the tabulated ones (table 2) indicating that no significant difference was observed regarding both accuracy and precision at 95% confidence level between the two methods. The proposed method was found to be more advantageous by being much more sensitive and less time consuming.

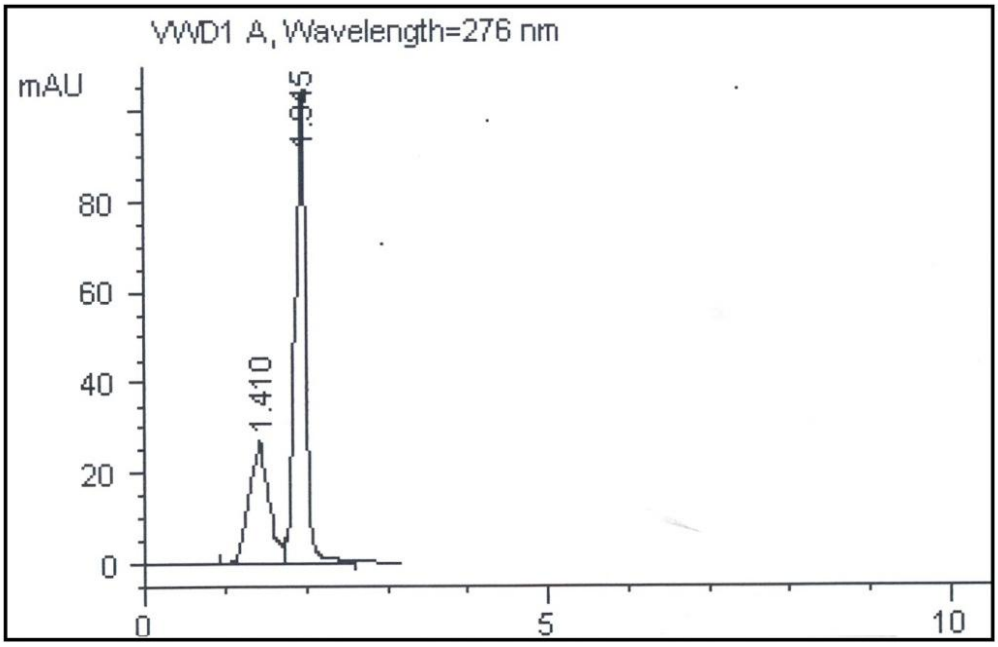


Fig. 1: Typical HPLC Chromatogram of Gf (100µgml^{-1}) and acidic degradation (100µgml^{-1}) on ODS column using a mobile phase consists of acetonitril: methanol: ammonium dihydrogen phosphate 0.005% (60: 30: 10 by volume), flow rate of 0.8 $mlmin^{-1}$ and UV detection at 276 nm.

TLC method

Recently, several TLC-UV densitometric methods had been successfully used for the determination of many drugs in presence of their degradation products [30]. Screening of the literature revealed that no densitometric methods concerning the determination of Gf and Ff. Thus, the development of a method for the determination of them in presence of their degradation products is of great importance. In the present work; stressed hydrolytic degradation was performed to study Gf& Ff stability in acidic and alkaline media via refluxing in different concentrations of NaOH & HCL at different time intervals and testing with TLC for complete degradation. Different concentrations of HCL were tried and 7 M HCl and heating for 3 hours were capable to produce complete hydrolysis of Gf and 7 M HCl and 7 M NaOH heating for 7 hours were capable to produce complete acidic and basic hydrolysis for Ff. A representative chromatogram is shown in fig.2 (A & B). Scan mode and wave length of detection were optimized as mentioned under construction of calibration curve to provide accurate, precise and reproducible results. Correlations were obtained between peak areas and concentrations of the two drugs in the range of 4-20µg/spot for Gf and 1-9µg/spot for Ff. The proposed TLC method is valid and applicable for the determination of intact Gf and Ff and the selectivity was proved by the determination of laboratory prepared mixtures containing different ratios of the drugs and their degradation products and it was found to be valid up to 80% for Gf and up to 90% for Ff of degradation products, tables (1 & 3). The proposed methods were successfully applied for the determination of the cited drugs in their tablets (Lopid and Fenorate). The validity was assessed by applying the standard addition technique. The small relative standard deviations indicate precision of the method. The results obtained by the proposed method were also compared with the official methods [13, 25] for Gf and Ff; the calculated t & F values were less than the tabulated ones (table 2& 4). Therefore no significant difference was observed regarding both accuracy and precision at 95% confidence level between the two methods. The proposed method is more advantageous by being much more sensitive and selective.

Two acid degradation products of Gf were carefully separated from a preparative TLC silica gel F_{254} plates, dissolved in methanol filtered and evaporated. The residues were subjected to IR and MS spectroscopic analysis to elucidate the structures. The assignment of the structures were based on comparison of IR and MS data of the purified specimen separated from the degradation reaction, with that of the intact compound. Briefly, it was found that IR of the first acid degradate of Gf C=C stretching appeared which did not appear in that of intact which prove our suggestion (Scheme 1, A). MS clarified that the second acid degradate of Gf had a larger molecular weight than that of intact. The same procedures were done to acid and base degradation of Ff; in the IR of the first acid degradate appeared –OH binding which did not appear in that of intact and the second acid degradate of Ff was the same as that of the first acid degradate but increased one molecule of water and one molecule of HCl which was verified by MS; by the increase in molecular weight. Regarding the basic degradate of Ff the disappearance of C=O of ketone signal which is found in IR of the intact was the principle of our suggestion (scheme 1, B).

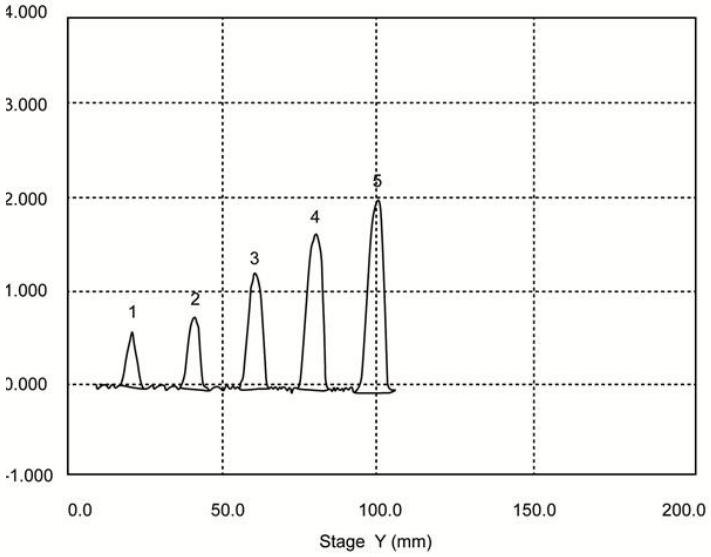


Fig. 2 (A): Typical TLC Chromatogram of 4-20 µ/spot Gf using TLC sheets of silica gel 60 F_{254} , ethyl acetate: methanol: 25%NH3 (17: 10: 1 by volume) as mobile phase.

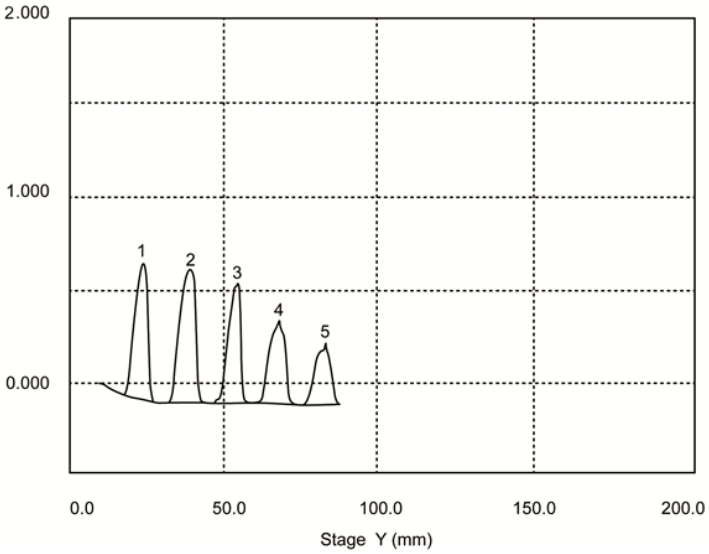
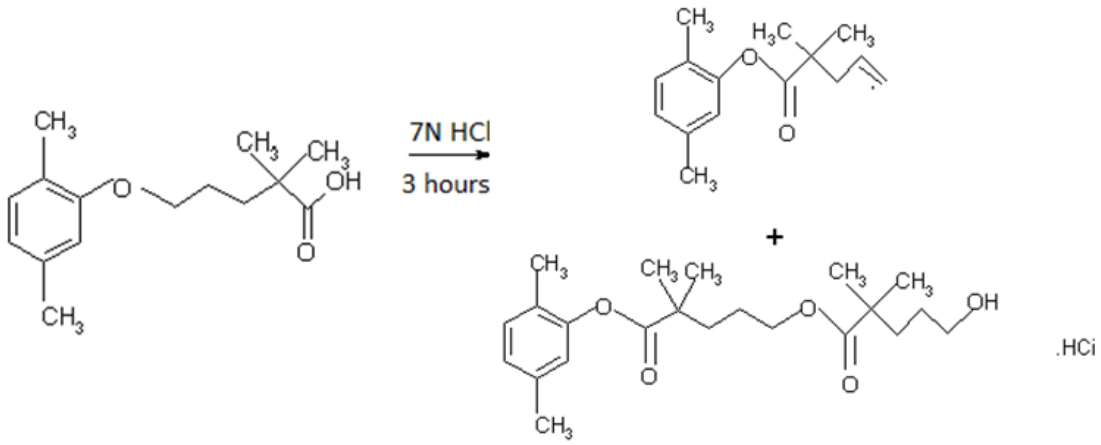
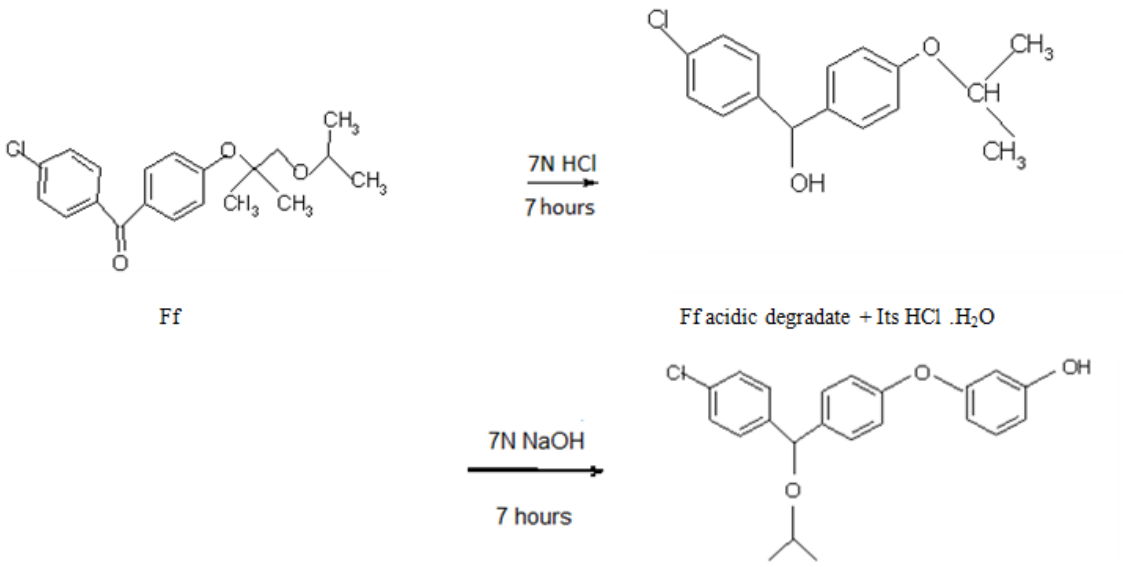


Fig. 2 (B): Typical TLC Chromatogram of 9-1 µ/spot Ff using TLC sheets of silica gel 60 F_{254}, tetrahydrofurane: hexane (5:6 by volume) as mobile phase.



Gf Gf acidic degradates

Scheme 1A



Scheme 1B

Fluorimetric method

Spectrofluorimetry was applied in the field of biomedical studies, including clinical, pharmacological and pharmaceutical analysis, because of its higher sensitivity and degree of specificity [7, 8]. In the present work Gf showed native fluorescence in N/10 NaOH with maximum emission at 405 nm when excited at 300 nm, and ~ zero measurement of its degradation (Fig. 3). Different factors affecting the fluorescence intensity have been investigated and optimized in order to improve sensitivity and selectivity of the method. *Solvents;* different solvents including methanol, N/10 meth. NaOH, N/10 NaOH, N/10 H_2SO_4, acetone, propanol, ethanol, acetonitrile were tried. Fluorescence intensity was recorded using appropriate blank at $\lambda_{em.}$ at 405 nm and $\lambda_{ex.}$ at 300nm. As shown in Fig.4 N/10 NaOH was the solvent of choice. *Excitation wavelength;* different excitation wavelengths were tested it was found that $\lambda_{em.}$ at 405 nm and $\lambda_{ex.}$ at 300 nm gives the best fluorescence intensity. *Time*; fluorescence intensity was observed up to two hours using 10 minutes interval, gradual increase from 70 to 120 minutes was found and maximum fluorescence intensity was reached after 100 minutes (Fig. 5). *Surfactants*; different surfactants such as cationic (cetyl pyridinium chloride), nonionic (tween 80) and anionic (SLS) were tried but none of them was used because cationic and nonionic were lowering the intensity and anionic has no effect.

Upon applying the proposed method, a linear relationship between the fluorescence intensity and the corresponding concentration of Gf in the range of 0.1-1.1µg ml^{-1} was found. Concentration was calculated from the following regression equation

F.I. = 749 C + 23.76 r = 0.9999

Where F.I. is the fluorescence intensity, C is the concentration in μgml^{-1} and r is the correlation coefficient. The mean percentage recovery for analyzing pure samples of Gf was calculated and found to be 100.74 ± 0.94.

In order to demonstrate the validity and the applicability of the proposed spectrofluorimetric method, as stability indicating method recovery studies were performed by analyzing laboratory prepared mixtures containing different ratios of Gf acid degradate (up to 90%) as shown in Table1. The results obtained by the proposed method were compared with the official HPLC method [13]. The calculated t & F values were less than the tabulated ones (table 2), therefore no significant difference was observed regarding both accuracy and precision.

The high sensitivity attained by the proposed spectrofluorimetric method allows the determination of Gf in spiked human plasma. The previously mentioned procedure under *Procedure of spiked human plasma* was applied and the concentration of GF was calculated from the following regression equation

F.I. = 650.7 C -1.262 r = 0.999.

Where F.I. is the fluorescence intensity of Gf, C is the corresponding concentration μgml^{-1} and r is the correlation coefficient. The proposed method is much more advantageous by being much more sensitive and less time consuming.

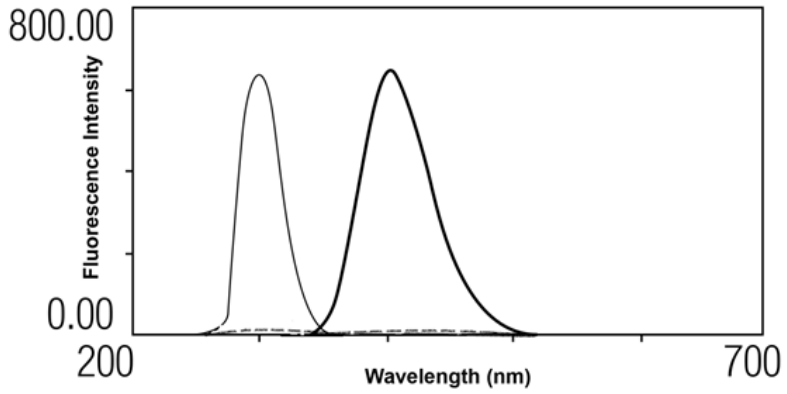


Fig. 3: Excitation and emission Spectra of intact Gf (———) and acidic degradation (- - - - -) ($0.9\mu ml^{-1}$) using 0.1N NaOH as solvent where $\lambda_{em.}$ 405 nm and λ_{ex} 300 nm.

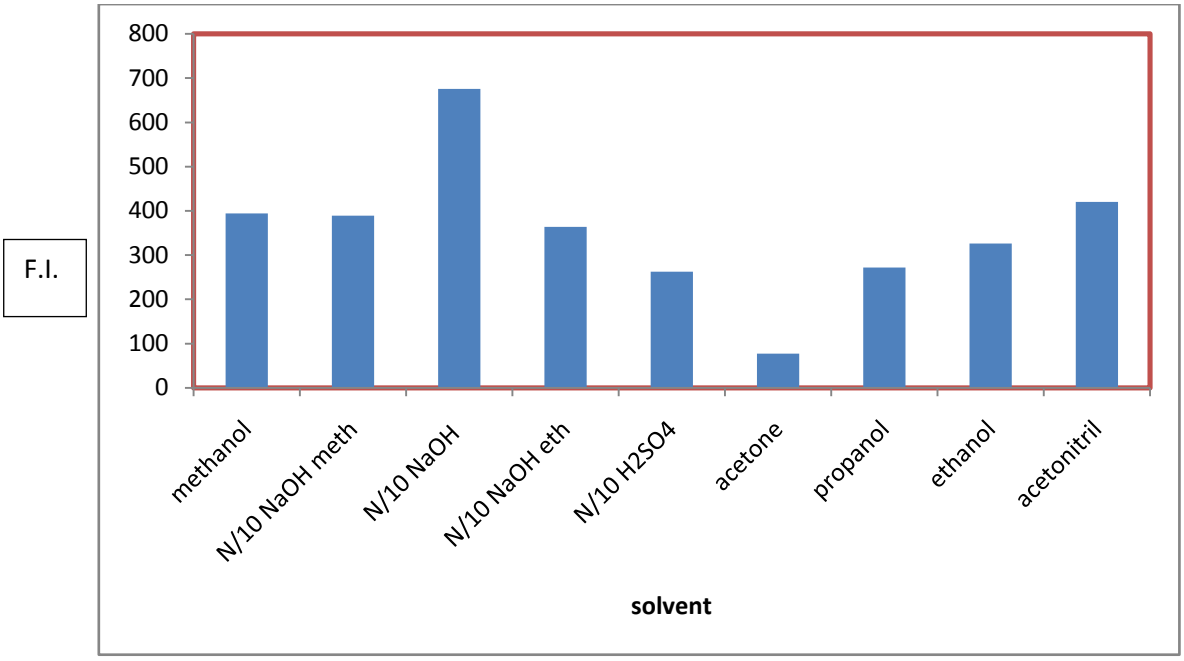


Fig. 4: Effect of solvent on fluorescence intensity.

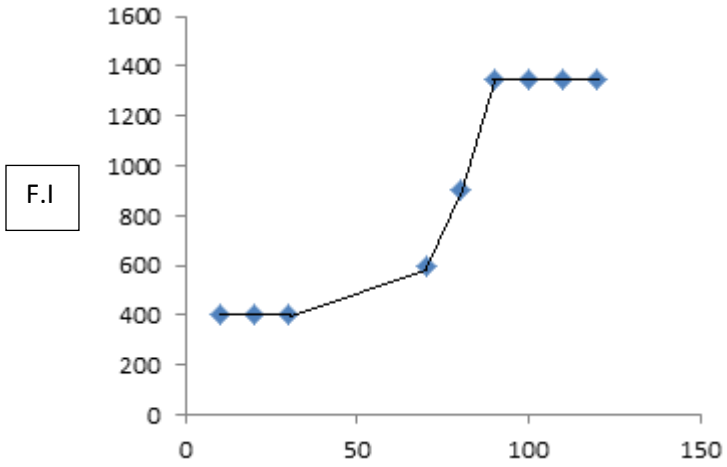


Fig. 5: Effect of time on fluorescence intensity.

1D method

Derivative spectrophotometric method offers greater selectivity in the analysis of drugs either in binary, ternary mixture or in the analysis of the intact drug without previous chemical separation [24, 31]. Zero-order absorption spectra of Ff and its degradates in methanol showed a large interference. Measuring the peak amplitudes of the first derivative curves at 248.4nm and at 250.8nm for the acidic and basic degradation products; respectively at zero contribution of their degradation products were used for its determination (Fig. 6 A & B). The main parameters that affect the shape of the derivative spectra which are the wavelength, scanning speed, the wavelength increment over which the derivative is obtained (Δ λ) and the degree of smoothing were optimized to give a well resolved peaks. Linear relationship was obtained between the peak amplitude and the concentration over the range of 2-12μg^{-1}ml for both acidic and basic degradates of Ff, from which the linear regression equations were computed and were found to be

$_1$D Ff a =0.058C+0.028 r=0.9987 at λ= 248.4 nm.

$_1$D Ff b =0.0911C+0.0383 r=0.9995 at λ =250.6 nm.

Where, $_1$D Ff a and $_1$D Ff b are the peak amplitude of first derivative spectra of Ff in presence of acidic and basic degradations; respectively, C is the corresponding concentration μgml^{-1} and r is the correlation coefficient. The selectivity and specificity of the proposed method was proved by the analysis of laboratory prepared mixtures containing different ratios of the drug and its acid and basic-induced degradation products. It was found to be valid up to 40% of acid degradation products; while in case of basic degradation product it was found to be valid up to 75% (table 3). The accuracy of the proposed method was evaluated by applying the proposed $_1$D method and the results obtained were compared with the official HPLC method [25]; The calculated t & F values were less than the tabulated ones (table 4), therefore no significant difference was observed regarding both accuracy and precision.

The proposed methods were successfully applied for the determination of Gf & Ff in their pharmaceutical dosage form. The results obtained were statistically compared with the official HPLC methods [13, 25] and no significant difference was obtained (table 5, 6).

The developed chromatographic (HPLC, TLC) methods are simple, accurate, precise, selective and readily adaptable to both pure drug and pharmaceutical dosage form without either pretreatment or preliminary separation and without interference from excepients and degradation products. The spectrophotometric method is quite simple and rapid. The fluorimetric method in addition to its sensitivity could be applied to human plasma.

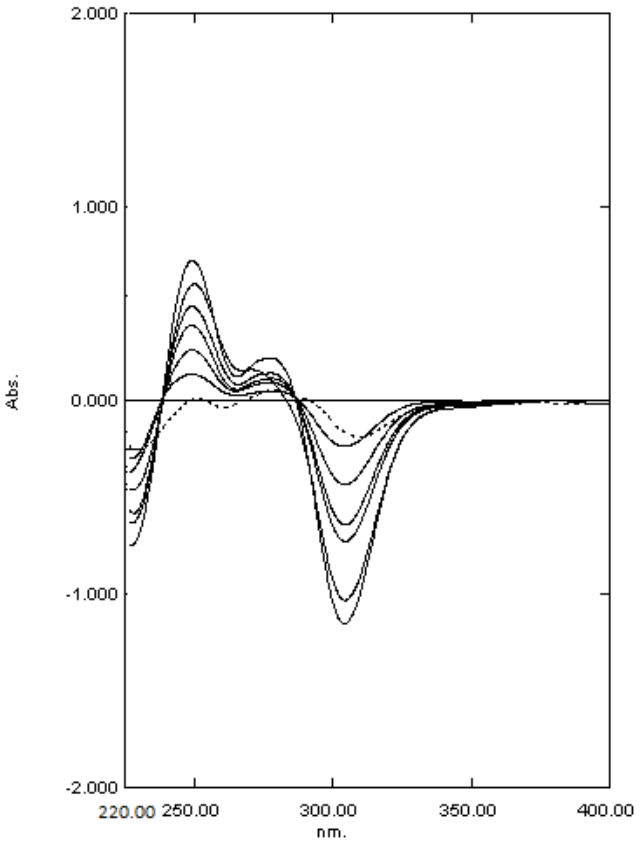


Fig. 6 (A): The first derivative curve at 248.4 nm 2-12 µgmL^{-1} Ff (———) and 10 µgmL^{-1} acidic degradation (- - - - -).

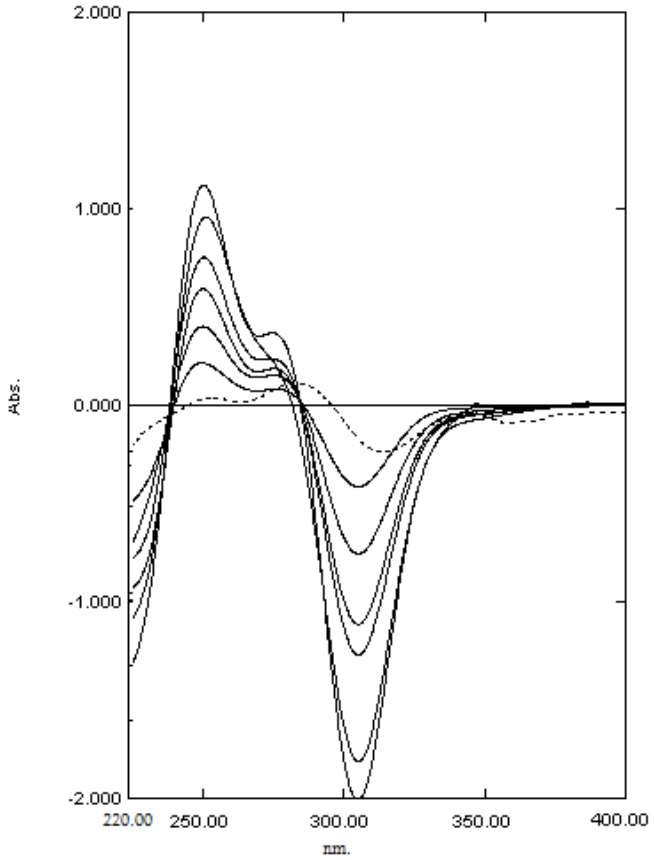


Fig. 6 (B): The first derivative curve at 250.8 nm 2-12 µgmL^{-1} Ff (———) and 10 µgmL^{-1} basic degradation (- - - - -).

Table 1: Determination of Gf in laboratory prepared mixtures by the proposed methods.

Sample Number	HPLC		TLC		Fluorimetric	
	Degradation %	Recovery %*	Degradation %	Recovery %*	Degradation %	Recovery %*
1	20	99.7	20	102	10	98
2	30	101	30	98	20	100.2
3	40	99.8	40	100.2	30	100.7
4	50	100.9	60	102	50	100.9
5	70	100.7	70	101.4	60	101.2
6	80	102	80	98	70	100.9
7					90	102
Mean ± RSD		100.68±0.84		100.26±1.86		100.56±1.25

*Average of three experiments.

Table 2: Statistical comparison of the results obtained by applying the proposed HPLC, TLC and fluorimetric methods and the official method for the analysis of pure Gf.

Value	HPLC		TLC		Fluorimetric	
	Proposed	Official Method[13]	Proposed	Official Method[13]	Proposed	Official Method[13]
Mean	99.9	100.18	100.5	100.18	99.28	100.18
S.D.	1.9	1.26	1.19	1.26	1.56	1.26
Variance	3.61	1.59	1.42	1.59	2.43	1.59
N	3	3	3	3	4	3
F-value ( )	2.25(5.79)		2.28(5.79)		1.53(5.41)	
Student's t test( )	0.27(2.78)		0.24(2.78)		0.84(2.57)	

Values in parenthesis are the theoretical values of t and F at P= 0.05.

Table 3: Determination of Ff in laboratory prepared mixtures by the proposed methods.

	TLC			$_1$D		
Sample Number	Degradation %	Recovery%* Acidic degradate	Recovery%* Basic degradate	Degradation %	Recovery%* Acidic degradate	Recovery%* Basic degradate
1	10	99	98	10	99	101
2	30	101	98.5	20	99.75	101
3	50	99.5	99	30	99.6	99.8
4	70	98	101.5	40	102	98
5	90	101.5	102	60	102	
				75	102	
Mean ± RSD		99.80±1.40	99.80±1.82		101.00 ± 1.41	99.90 ± 1.42

*Average of three experiments.

Table 4: Statistical comparison of the results obtained by applying the proposed TLC and $_1$D method and the official method for the analysis of pure Ff.

Value	TLC		$_1$D		
	Proposed Method	Official Method[25]	Acidic degradate	Basic degradate	Official Method[25]
Mean	99.7	101	100	99.5	101
S.D.	1.74	1.51	1.15	0.84	1.51
Variance	3.03	2.28	1.32	0.71	2.28
N	3	3	3	3	3
F-value	1.33(5.79)		1.72(5.79)	3.21(5.79)	
Student's t test	0.98(2.78)		0.91(2.78)	1(2.78)	

Values in parenthesis are the theoretical values of t and F at P= 0.05.

Table 5: Results obtained by applying the proposed methods for the determination of Gf in its pharmaceutical formulations.

Preparation	HPLC			TLC			Fluorimetry		
	Taken (μgml^{-1})	Found* (μgml^{-1})	Recovery %	Taken (μg/spot)	Found * (μg/spot)	Recovery %	Taken (μgml^{-1})	Found* (μgml^{-1})	Recovery %
	6	6.03	100.5	6	6.06	101	0.2	0.2	101.6
Lopid tablet B. N. 9105	10	10.2	102	8	8.02	100.2	0.4	0.39	99
600mg/tablet	12	11.76	98	12	12.12	101	0.6	0.6	100.2
	14	13.3	99	14	14.38	102.7	0.8	0.8	100
Mean ± RSD			98.88±1.75			101.22±1.05			100.20±1.07

*Average of three experiments.

Table 6: Results obtained by applying the proposed methods for the determination of Ff in its pharmaceutical formulations.

Preparation	TLC			$_1$D acidic degradate			$_1$D basic degradate	
	Taken (µg/spot)	Found * (µg/spot)	Recovery %	Taken (µgml^{-1})	Found * (µgml^{-1})	Recovery %	Found* (µgml^{-1})	Recovery %
Fenorate tablet B.N. 5571 250 mg/tablet	5	5.1	102	4	3.96	99	3.92	98
	7	7.07	101	6	5.88	98	5.94	99
	9	8.91	99	8	7.84	98	7.89	98.6
				10	9.9	99	9.83	98.3
				12	12.24	102	12.24	102
Mean ± RSD			100.67±1.53			99.20±1.62		99.18±1.62

*Average of three experiments.

CONCLUSION

The proposed HPLC, TLC, Fluorimetry and 1D methods are advantageous over the manufacturer and published methods regarding sensitivity and simplicity. Moreover, these methods determine gemfibrozil and fenofibrate in drug substance, drug products without interference from excipients and degradation products. The HPLC, TLC are accurate, precies and simple, Fluorimetry is much more sensitive. The spectrophotometric method is sufficiently simple and rapid.

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