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Stability-indicating methods for the determination of famciclovir in the presence of its alkaline-induced degradation product

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Five sensitive, selective and precise stability-indicating methods are presented for the determination of famciclovir (FCV) in the presence of its alkaline-induced degradation product. Method A utilizes the first derivative spectrophotometry at 321 nm. Method B depends on using the first derivative of the ratio spectrophotometry (DD^1) by measurement of the amplitude at 256 nm. Method C is based on the reaction of FCV with hydroxylamine to form hydroxamic acid, causing the hydroxamic acid to react with triferric ion to form ferric hydroxamate that is measured at 503 nm. Method D is based on the separation of FCV from its degradation product followed by densitometric measurement of the bands at 304 nm. The separation was carried out on silica gel 60 F_{254}, using chloroform: methanol (70 : 30, v/v) as a mobile phase. Method E is based on a high performance liquid chromatographic (HPLC) separation of FCV from its degradation product using an ODS column with a mobile phase consisting of methanol – 50 mM dipotassium hydrogen phosphate (25 : 75, v/v, pH 3.0)with UV detection at 304 nm. Regression analysis showed good correlation in the concentration ranges 16 – 72 μg/ml, 40 – 240 μg/ml, 40 – 240 μg/ml, 0.75 – 5.25 μg/band and 20 – 240 μg/ml with percentage recoveries of 99.65 ± 0.85, 100.27 ± 0.91, 99.72 ± 0.84, 100.65 ± 1.52 and 99.88 ± 0.50 for methods A, B, C, D and E, respectively. These methods are suitable as stability-indicating methods for the determination of FCV in the presence of its degradation product either in bulk powder or in pharmaceutical formulation. Statistical analysis of the results has been carried out revealing high accuracy and good precision.

Keywords: famciclovir; derivative spectrophotometry; ferric hydroxamate; densitometry; HPLC; stability-indicating method

Introduction

Chemically, famciclovir (FCV), known as 9-[4-Acetoxy-3-(acetoxymethyl) but-1-yl]-2-aminopurine[1] (Figure 1), is a novel antiviral drug, which is highly efficient in the treatment of acute uncomplicated herpes zoster.[2] FCV is a synthetic guanine derivative, which is metabolized to penciclovir which has the potent antiviral activity as another 9- substituted guanine derivative like acyclovir. Penciclovir is active against herpes simplex virus Type 2, vericella zoster virus I, Epstein-Barr virus and hepatitis B.[3] FCV is absorbed rapidly and extensively after oral administration, and total systemic availability of penciclovir is 77%.[4] Metabolism of FCV involves sequential hydrolysis of both acetyl groups to give 6-deoxypenciclovir, which is subsequently oxidized to penciclovir.[5]

FCV is not official in any Pharmacopoeia. Few methods have been reported for the quantitative estimation of FCV in pharmaceutical formulations and in body fluids. These methods include derivative method,[6] colorimetric methods using Tpooo (benzene sulfonic acid),[7] Folin's reagent and resorcinol,[8] bromocresol green, bromothymol blue, bromocresol purple and bromophenol blue,[9] 2,6- dichloroquinone chlorimide,[10] high performance liquid chromatographic (HPLC) methods,[6,11 – 15] capillary electrophoresis,[16] electrochemical method[17] and electro-spray ionization mass spectrometry.[18]

To date, no simple stability-indicating analytical method has been described in the literature, and no previous systematic studies focused on FCV degradation have been performed. The goal of the present study was to develop and validate simple stability-indicating methods to be used for quality control of FCV in pharmaceutical preparations. These methods include first derivative (D^1), first derivative of the ratio spectra (DD^1) and ferric hydroxamate method, as well as chromatographic methods, namely, TLC-densitometry method and HPLC.

The scientific novelty of the present work is that the methods used are simple, rapid, selective, less expensive and less time-consuming compared to published methods.

Experimental

Instruments

A double beam UV/VIS spectrophotometer (Shimadzu, Japan) model UV- 1650 PC with a quartz cell of 1 cm path length. The spectral band width is 2 nm and the wavelength-scanning speed is 2800 nm/min.

An IR spectrophotometer (Shimadzu 435, Kyoto, Japan); sampling was undertaken as potassium bromide discs.

A mass spectrophotometer: MS-QB 1000 EX, Finnigan Nat (USA).

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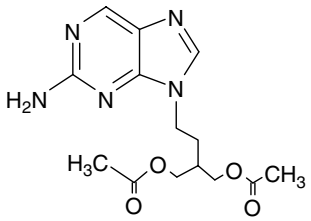


(M. Wt = 321.3)

Figure 1. Structural formula of Famciclovir.

TLC-plates (20 cm × 20 cm) coated with silica gel 60 F_{254} (Merck, Germany).

TLC scanner 3 densitometer (Camag, Muttenz, Switzerland).

The following requirements are taken into consideration:

- Source of radiation: deuterium lamp
- Slit dimensions: 3 mm × 0.45 mm
- Scan mode: absorbance mode
- Scanning speed: 20 mm s^{-1}
- Output: Chromatogram and integrated peak area

A sample applicator for TLC Linomat IV with 100 µl syringe (Camag, Muttenz, Switzerland).

A liquid chromatograph consisting of an isocratic pump (Agilent Model G1310A), an ultraviolet wavelength detector (Model G1314A, Agilent 1100 Series), a Rheodyne injector (Model 7725 I, USA) equipped with 20 µl injector loop, An Agilent (USA) column Zorbax 10 µm C_{18} (150 mm × 4.6 mm.), a Teflon membrane filter of pore size 0.45 µm and 47 mm diameter for solvent and a Teflon disposable membrane filter of pore size 0.45 µm for samples.

Materials

Pure standard

FCV was kindly supplied from Kahira Pharm. & Chem. Ind. Co., Cairo, Egypt (Egypt). Its purity was found to be 99.97%, according to a reported HPLC method.[6]

Pharmaceutical dosage form

Famclovir® tablets are labelled to contain 200 mg FCV manufactured by Kahira Pharm. & Chem. Ind. Co. for Multipharma Co. Cairo, Egypt (Egypt). Batch No. 0611292 and 0611293.

Degraded sample

Hundred (100) milligrams of FCV were refluxed with 100 ml of 2M NaOH solution for 15 min. Complete degradation was confirmed by TLC through the disappearance of drug spot using chloroform : methanol (70 : 30 v/v) as a developing system. The solution was neutralized with 2M HCl, evaporated to small volume, diluted to 10 ml with methanol. The degradation product was separated on preparative TLC plates using chloroform: methanol (70 : 30, *v*/*v*) as a developing system. The band corresponding to degradation product was scratched, dissolved in methanol. The solution was stirred, filtered and the solvent was allowed to evaporate. The separated degradation product was subjected to IR and mass spectral analyses for subsequent identification.

Chemicals and solvents

All chemicals used throughout this work were of analytical grade and the solvents were of spectroscopic grade.

1. Methanol (Spectr oscopic grade & HPLC grade) Pennsylvania, USA (Aldrich-USA)
2. Chloroform and dipotassium hydrogen phosphate (Al-Gomhoria-Egypt)
3. Hydroxylamine hydrochloride (Oxford-USA).
4. Ferric chloride (Aldrich-USA)
5. Conc. HCl and NaOH pellets (Adwic-Egypt)

Reagents

Hydroxylamine hydrochloride (20%)**:** 20 g of hydroxylamine hydrochloride was dissolved in 100 ml distilled water. Ferric chloride (6%) was freshly prepared by dissolving 6 g of ferric chloride in 100 ml distilled water. HCl (2M& 4 M) solutions were prepared in distilled water. NaOH (2M& 4 M) solutions were prepared in distilled water. 50 mM dipotassium hydrogen phosphate was prepared in distilled water.

Stock standard solutions

Stock standard solutions of FCV and its alkaline degradation product (0.8 mg/ml) in methanol (for D^1 method). Stock standard solutions of FCV and its alkaline degradation product (1 mg/ml) in methanol (for DD^1 method), in distilled water (for colorimetric method) and in methanol HPLC grade (for HPLC method). Stock standard solutions of FCV and its alkaline degradation product (0.15 mg/ml) in methanol (for TLC-densitometric method).

Laboratory prepared mixtures

Solutions containing different ratios of FCV and its alkaline degradation product were prepared to contain 10–90% of alkaline degradation product of FCV.

Procedures

Construction of calibration graph for D^1 spectrophotometric method

A series of working standard solutions containing 16–72 µg/ml FCV were prepared separately in methanol using its corresponding standard solution (0.8 mg/ml). The first derivative absorption spectra of the UV spectrum of each solution against methanol as blank were recorded. D^1 curves were recorded at $\Delta\lambda = 2.00$ and scaling factor =8. The calibration graph was obtained by plotting the peak amplitudes at 321 nm (corresponding to zero-contribution of the degradation product) of D^1 spectra versus the corresponding concentrations of FCV, and the regression equation was computed.

Construction of calibration graph for DD^1 spectrophotometric method

A series of working standard solutions containing 40–240 µg/ml FCV were prepared in methanol using its corresponding standard solution (1 mg/ml). The absorption spectra of the resulting solutions were measured and divided by the absorption spectrum of 40 µg/ml degradation product (as a divisor), and then the obtained ratio spectra were differentiated with respect to wavelength. First derivative values ($\Delta\lambda = 2.00$, scale factor =8)

at 256 nm were recorded. The calibration graph representing the relationship between the measured amplitudes and the corresponding concentrations of FCV in µg/ml was constructed, and the regression equation was computed.

Construction of calibration graph for ferric hydroxamate method

Aliquots of FCV ranging from 1–6 ml were transferred into a series of 25 ml calibrated volumetric flasks using its corresponding standard solution (1 mg/ml). To each 2 ml of hydroxylamine hydrochloride solution (20%) and 4 ml of 4M NaOH were added at room temperature. After 1 min, 4 ml of 4M HCl and 4 ml of $FeCl_3$ solution (6%) were added. The volumes were made up to the mark with distilled water. The absorbance of the coloured chromogen was measured at 503 nm against a reagent blank. The calibration graph representing the relationship between the absorbance at 503 nm and the corresponding concentrations of FCV in µg/ml was constructed and the regression equation was computed.

Construction of calibration graph for TLC densitometric method

Aliquots equivalent to 0.75–5.25 µg were applied in the form of bands on a TLC plate using a Camag Linomat IV applicator. The band length was 4 mm and dosage speed was 150 nl s^{-1}. The bands were applied 14 mm apart from each other and 10 mm from the bottom edge of the plate. Linear ascending development was performed in a chromatographic tank previously saturated for 1 h with the developing mobile phase consisting of chloroform: methanol (70 : 30 *v*/*v*) at room temperature. The plates were developed over a distance of 15 cm and air dried at room temperature. FCV was scanned at 304 nm. The integrated peak areas were recorded. The calibration curve was constructed by plotting the integrated peak areas versus the corresponding concentrations of FCV in µg/band and the regression equation was computed.

Construction of calibration graph for liquid chromatographic method

A series of working standard solutions containing 20–240 µg/ml FCV were prepared using its corresponding standard solution (1 mg/ml) in the mobile phase. The samples were filtered through 0.45 Teflon membrane filter prior to analysis. Triplicates 20-µl injections of each solution were made and chromatographed using a Zorbax 10 µm C_{18} column (150 mm × 4.6 mm). Separation was achieved using methanol–50 mM dipotassium hydrogen phosphate (25 : 75, v/v, pH 3.0) as a mobile phase at a flow rate of 1 ml/min and UV detection of effluent at 304 nm. The column was maintained at ambient temperature (~25 °C). The mobile phase was filtered through 0.45 µm Teflon membrane filter and degassed for ~15 min in ultrasonic bath prior to use. To reach good equilibrium, the analysis was usually performed after passing ~50–60 ml of the mobile phase, just for conditioning and pre-washing of the stationary phase. The peak areas were recorded and the average peak areas were calculated. The calibration graph was plotted representing the relative peak areas (average peak area of FCV to that of the external standard; 60µg/ml) against the corresponding concentrations of FCV in µg/ml and the regression equation was computed.

Analysis of laboratory prepared mixtures containing different ratios of FCV and its degradation product using the suggested methods

Proceed as mentioned under the calibration procedure of each method. The concentration of FCV was calculated from the corresponding regression equation.

Application to pharmaceutical formulation

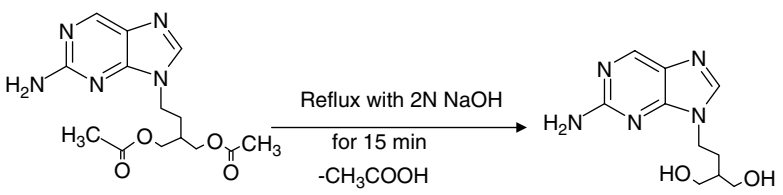
Twenty tablets of Famclovir were accurately weighed and finely powdered. An accurately weighed amount of the powder equivalent to 100 mg of FCV of each was transferred into a series of 100-ml volumetric flasks and 75 ml of the appropriate solvent was added (methanol for D[1], DD[1] and TLC-densitometric methods, methanol HPLC grade for HPLC method and distilled water for ferric hydroxamate method), stirred well for 30 min and completed to the volume to obtain 1 mg/ml stock solutions, then filtered. The solutions were diluted to the same concentrations of the appropriate working solutions and proceed according to the calibration procedure of each method mentioned above. The concentration of FCV was calculated from the corresponding regression equation.

Results and Discussion

Following an oral administration in human, metabolism of FCV begins. The main circulating metabolite is 6- deoxypenciclovir, which is subsequently oxidized to penciclovir.[5]

FCV was subjected to acid and alkaline hydrolysis, oxidation degradation. The drug was found to be degraded under basic condition, the 6-deoxypenciclovir was obtained, and being inactive[3,5,19] the determination of FCV in the presence of its alkaline degradation product was essential.

The International Conference on Harmonization (ICH) guideline *Stability testing of new drug substances and products* requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance.[20] An ideal stability-indicating method is the one that quantifies the standard drug alone and also resolves its degradation product.



Famciclovir (M. Wt = 321.3)

6- deoxypenciclovir (M. wt = 237.3)

Figure 2. Proposed scheme for preparing the degradation product.

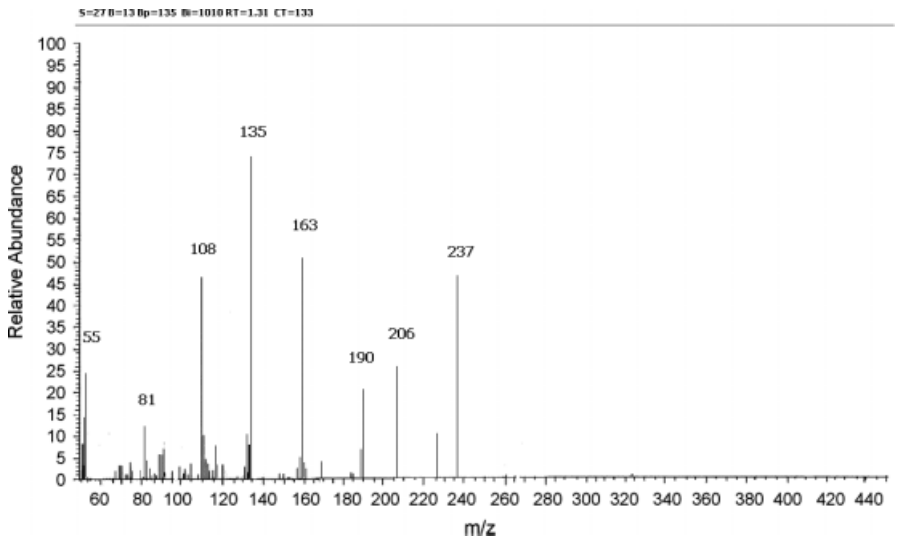


Figure 3. Mass spectrometry of the alkaline degradation product of Famciclovir.

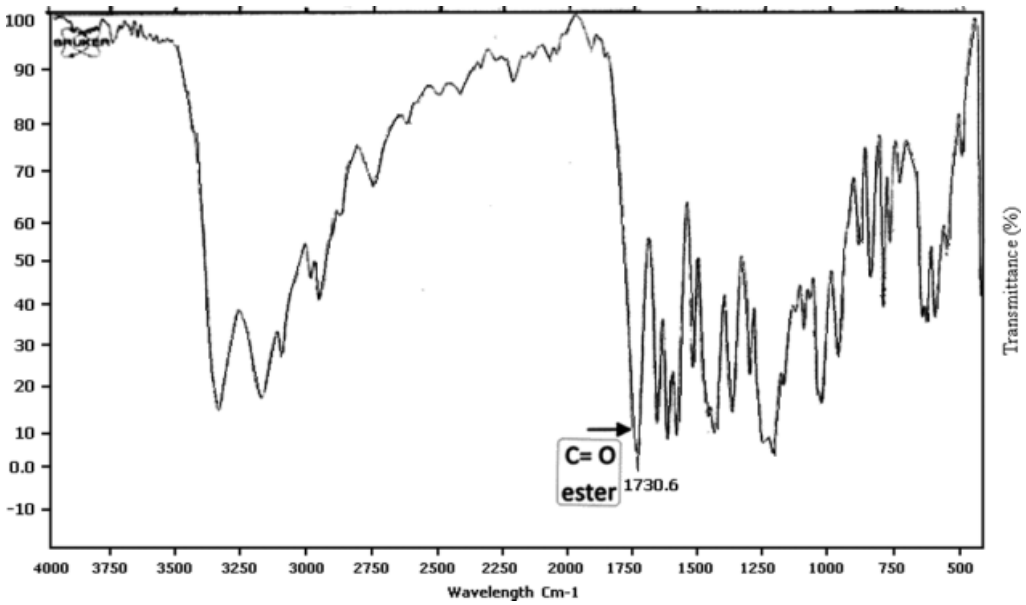


Figure 4. IR of intact Famciclovir.

Elucidation of the degradation product of FCV

FCV was subjected to forced degradation by refluxing with 2N NaOH for 15 min. (Figure 2). On the other hand, alkaline degradation product, 6-deoxy penciclovir, was isolated using preparative TLC plates and characterized by MS- and IR-spectrometry.

Mass spectrometry

In the MS chart, the parent peak was identified at *m/z* 237 (molecular weight of 6-deoxy penciclovir) (Figure 3). This proves that the prepared degradation product is the main degradation product. No other degradation products were observed under the conditions used to prepare 6-deoxypenciclovir.

Spectral changes

IR spectra show that the characteristic band at 1750–1730 cm^{-1} corresponding to ester group in the spectrum of intact FCV (Figure 4) disappeared in the IR spectrum of its alkaline degradation product (Figure 5); a broad band at 3550–3400 cm^{-1} of OH-alcohol appeared in the IR spectrum of the alkaline degradation product.

TLC-fractionation

TLC-monitoring of the drug degradation was done on thin layer plates of silica gel F_{254} using chloroform-methanol (70 : 30 *v/v*) as a developing system. The developed plates were visualized under short UV-lamp. The alkaline degradation product (R_f value = 0.48) could be separated elegantly from the intact drug (R_f value = 0.84).

Method Development

First derivative(D^1) method

The focus of the present work was to develop accurate, specific, reproducible and sensitive stability-indicating methods

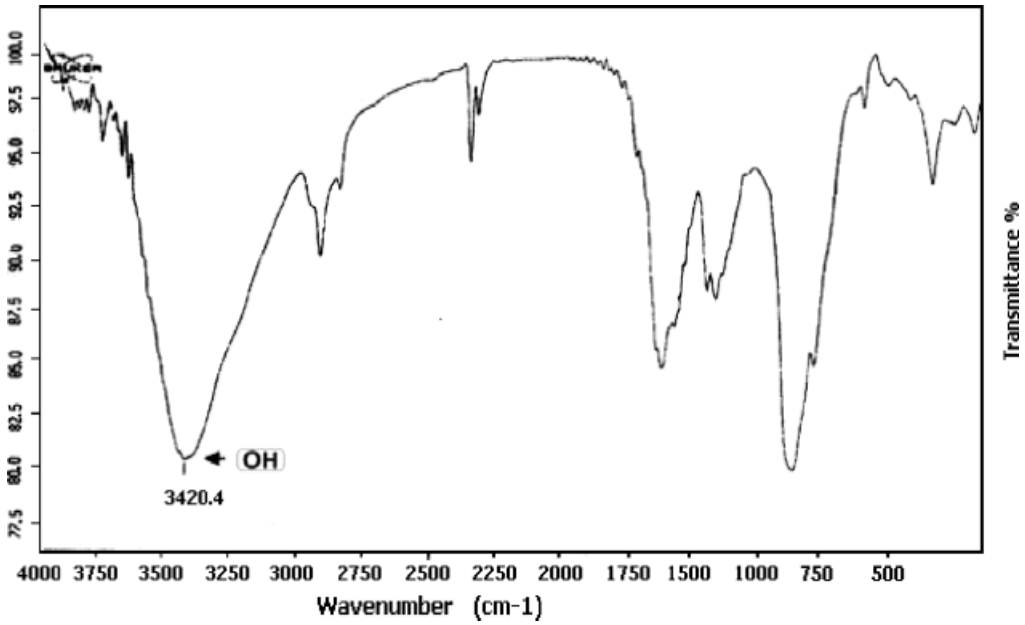


Figure 5. IR of the alkaline degradation product of Famciclovir.

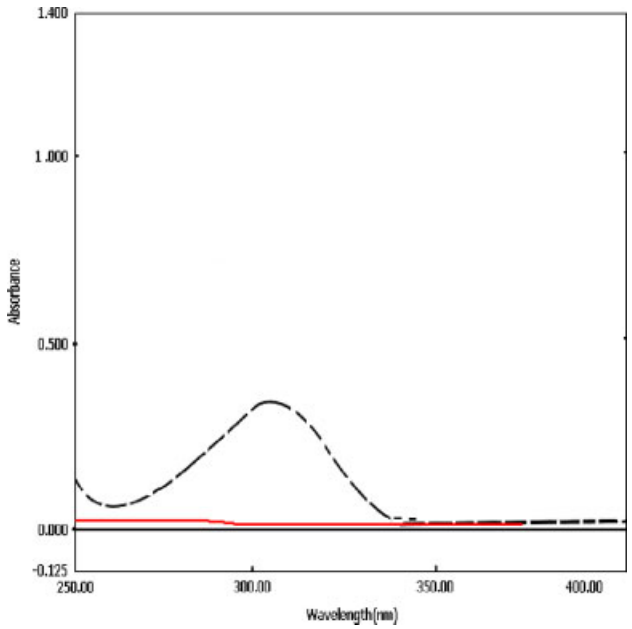


Figure 6. Zero-order absorption spectra of Famciclovir (- - - -) (16 µg/ml) and its degradation product (———) (16 µg/ml).

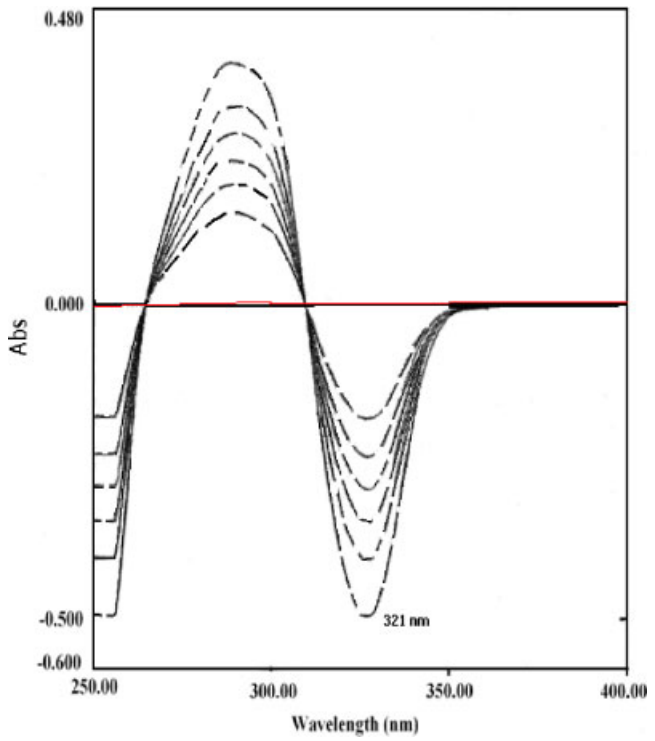


Figure 7. First derivative spectra of Famciclovir (16–72 µg/ml)(- - - -) and its degradation product (16 µg/ml) (———) in methanol.

for the determination of FCV in pure form or in pharmaceutical formulations without interference of its alkaline degradation product.

The zero-order absorption spectra of FCV and its alkaline degradation product showed certain overlapping (Figure 6) which interferes with the direct determination of FCV. Derivative spectrophotometry is an analytical technique of great utility for extracting both qualitative and quantitative information from spectra composed of unresolved bands, and for eliminating the effect of baseline shifts and baseline tilts by using the first or higher derivatives of absorbance with respect to wavelength.[21] A rapid, simple and low-cost spectrophotometric method based on measuring the peak amplitude of D^1 spectrum of FCV at 321 nm (corresponding to zero-contribution of the degradation product) was developed with good selectivity without interference of the alkaline degradation product as shown in Figure 7.

In order to optimize D^1 method, different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda = 2$ and a scaling factor = 8 showed a suitable signal to noise ratio and the spectra showed good resolution.

Table 1. Determination of FCV in laboratory-prepared mixtures by the proposed methods

Determination of intact Famciclovir in lab. mix.	Methods				
	D^1-method at 321 nm[a]	DD^1-Method at 256 nm[b]	Ferric hydroxamate Method[c]	TLC-densitometric Method[d]	HPLC method Method[e]
Mean ± S.D.	100.68 ± 1.15	99.35 ± 0.51	99.48 ± 0.87	100.16 ± 0.91	99.92 ± 1.42

[a,b] Up to 80% degradation product. [c] Up to 70% degradation product. [d,e] Up to 90% degradation product.

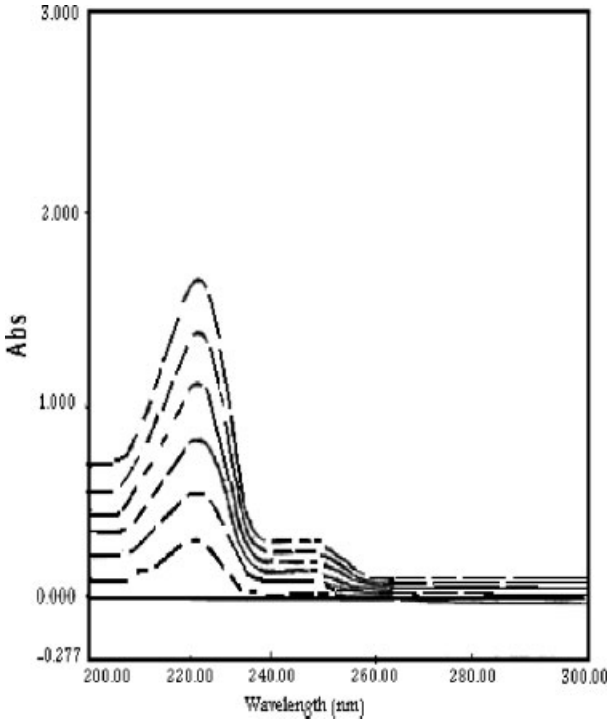


Figure 8. Zero-order of derivative ratio spectra of Famciclovir 40–240 µg/ml(- - - -) using 40 µg/ml of degradate as a divisor.

A linear correlation was obtained between peak amplitude at 321 nm and the corresponding concentration in the range of 16–72 µg/ml, from which the linear regression equation was computed and found to be

$$D^1 = 0.006C + 0.0789 \quad r = 0.9998 \quad \text{at } 321 \text{ nm} \qquad (1)$$

where D^1 is the peak amplitude at 321 nm, C is the concentration of FCV (µg/ml) and r is the correlation coefficient.

The proposed method is valid for the determination of FCV in the presence of up to 80% of its alkaline degradation product in different laboratory prepared mixtures as presented in Table 1, where at 321 nm showed higher selectivity with mean percentage recovery 100.68 ± 1.15.

First derivative of ratio spectra (DD^1) method

In order to improve the selectivity of the analysis of FCV in the presence of its alkaline degradation product, DD^1 spectrophotometric method was established. The main advantage of the method is that the whole spectrum of the interfering substance is cancelled. Accordingly, the choice of the wavelength selected for calibration is not critical as in the D^1 method.

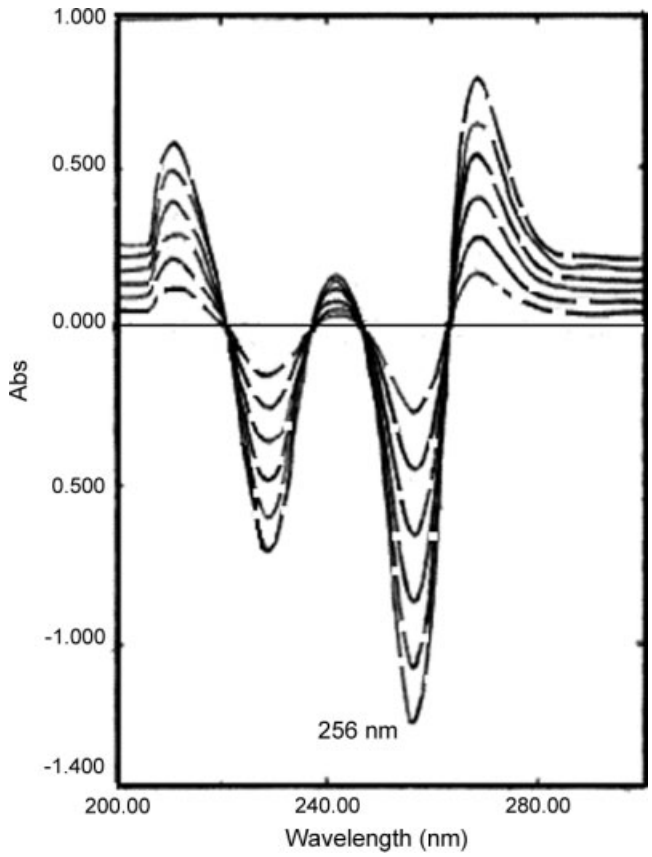


Figure 9. First derivative of ratio spectra of Famciclovir (40–240 µg/ml) using 40 µg/ml of alkaline degradate as a divisor.

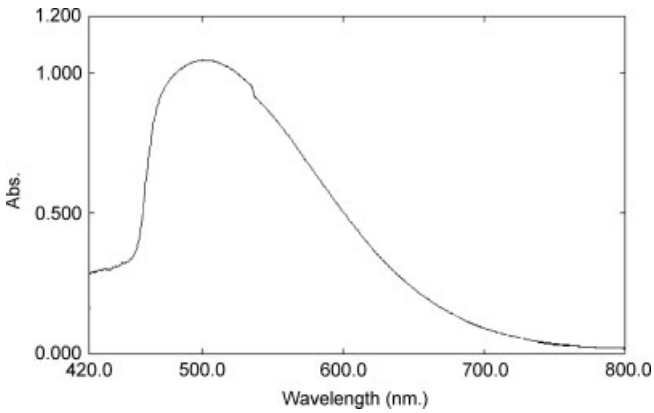


Figure 10. Ferric hydroxamate complex of FCV (220 µg/ml) at 503 nm.

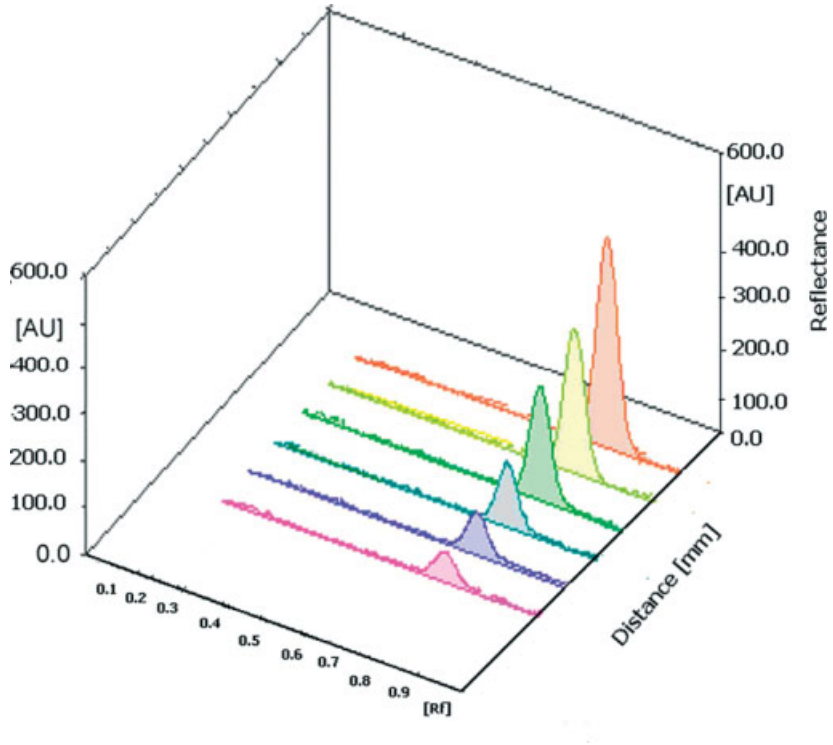


Figure 11. Scanning profile of TLC chromatogram of Famciclovir at 304 nm.

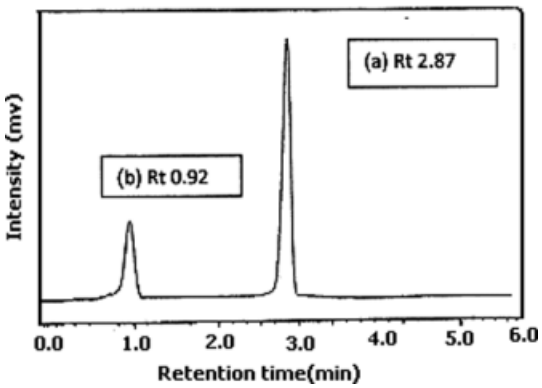


Figure 12. Liquid chromatographic separation of FCV(2.87 min) from its degradate (0.92 min), using the specified experimental conditions.

In order to optimize DD^1 method, several divisor concentrations (20, 40, 60 and 80 μg/ml) of the degradation product were tried, the best result was obtained when using 40 μg/ml of the degradation product as a divisor. Different smoothing and scaling factors were tested, where a smoothing factor $\Delta\lambda = 2$ and a scaling factor = 8 were suitable to enlarge the signal of FCV to facilitate its measurement and to diminish error in reading the signal (Figures 8 and 9).

Dividing the absorption spectra of FCV in the range of 40–240 μg/ml by the absorption spectrum of 40 μg/ml of the degradation product (as a divisor); the obtained ratio spectra were differentiated with respect to wavelength.

DD^1 values showed good linearity and reproducibility at 256 nm, the linear regression equation was computed and found to be

$$DD^1 = 0.005C + 0.0570 \quad r = 0.9996 \quad \text{at 256 nm} \qquad (2)$$

Table 2. Parameters required for system suitability test of HPLC method

Parameter	Obtained value	Reference value
Resolution (R)	3	R > 0.8
T (tailing factor)	FCV.: 1.0	T = 1 for a typical symmetric peak
	Deg. Product 0.938	
α (relative retention time)	5.64	>1
K' (column capacity)	FCV: 4.74	1–10 acceptable
	Deg. Product: 0.84	
N (column efficiency)	FCV.: 7413	Increases with efficiency of the separation
	Deg. Product: 564	
HETP	FCV.: 0.002	The smaller the value, the higher the column efficiency
	Deg. Product: 0.026	

FCV. : intact.
Deg. product : alkaline induced degradation product.
Peak width in cm.
Column length in cm.

where DD^1 is the peak amplitude of the first derivative curve for (FCV/its degradation product), C is the concentration of FCV (μg/ml) and r is the correlation coefficient.

The method was checked by analysis of laboratory-prepared mixtures of FCV and its alkaline degradation product in different ratios as presented in Table 1, where at 256 nm, FCV could be

determined in presence of up to 80%of degradation product with mean percentage recovery 99.35 ± 0.51.

Ferric hydroxamate method

The ferric hydroxamate method is widely used for the determination of esters[22–24] but it does not react with alcohols, so it can be used as a stability-indicating method for the determination of FCV in the presence of its alkaline degradation product.

Hydroxylamine, being unstable and easily oxidized by atmospheric oxygen, is never used alone. Thus, the hydroxylamine salt such as HCl-salt, which is a very stable reagent, is commonly used. To liberate HCl from hydroxylamine hydrochloride, the reaction should be carried out in alkaline medium, thus activating the lone pair of electrons present in the hydroxylamine base, essential for the reaction. Famciclovir reacts with hydroxylamine hydrochloride in alkaline medium (using 4M NaOH) forming hydroxamic acid. Upon acidification (using 4M HCl), and addition of Fe^{3+} ions, a purple-coloured product of ferric-hydroxamate is formed according to the following equations:

$$\mathrm{ROCOR' + :NH_2OH \longrightarrow R\text{-}CO\text{-}NHOH + R'OH} \quad (3)$$

$$\mathrm{3R\text{-}CO\text{-}NHOH + Fe^{3+} \longrightarrow Fe(R\text{-}CO\text{-}NHO)_3 + 3H^+} \quad (4)$$

The optimum conditions for the reaction were carefully studied. Maximum absorbance at 503 nm (Figure 10) was obtained immediately upon using 2 ml of hydroxylamine hydrochloride(20%), 4 ml of 4M NaOH, and 4 ml of $FeCl_3$(6%) at room temperature (25 ± 5 °C), to maintain the drug stability in alkaline medium, and the product remained stable for up to 30 min.

A linear correlation was obtained between the absorbance at 503 nm and the corresponding concentration in the range of 40–240 µg/ml, from which the linear regression equation was computed and found to be

$$A = 0.0058C - 0.0277 \quad r = 0.9998 \quad \text{at 503 nm} \quad (5)$$

where A is the absorbance at 503 nm and C is the concentration of FCV (µg/ml) and r is the correlation coefficient.

Results described in Table 1, show that this method is specific, valid and applicable for determination of FCV in presence of up to 70% of its degradation product with mean percentage recovery 99.48 ± 0.87.

TLC-densitometric method

The TLC-densitometric method technique offers a simple way to quantify directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing to a standard curve from reference materials chromatographed simultaneously under the same condition.[25]

To improve the separation of bands, it was necessary to investigate the effect of different variables. Studying the optimum parameters for maximum separation was carried out as follows:

Mobile phase. Different developing systems of different composition and ratios were tried for separation, e.g., chloroform-ethyl acetate (9.5 : 0.5, *v*/*v*), chloroform-methanol (5 : 5, *v*/*v*), chloroform-methanol (7.0 : 3.0, *v*/*v*), chloroform-methanol (9.5 : 0.5, *v*/*v*) and chloroform-methanol-ammonia (8 : 2:0.1, *v*/*v*/*v*). The best mobile phase was chloroform-methanol in ratio (7 : 3, *v*/*v*). This selected mobile phase allows the determination of FCV without interference from its alkaline degradation product and without tailing of the separated band (Figure 12).

Band dimensions. Different band dimensions were tested in order to obtain sharp and symmetrical separated peaks. The optimum band width chosen was 4 mm and the interspaces between bands were 14 mm.

Scanning wavelength. Different scanning wavelengths were tried; the optimum wavelength with higher sensitivity, symmetrical peaks and minimum noise was at 304 nm (λ_{max} of FCV).

Slit dimensions of scanning light beam. The slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried, where 3 mm × 0.45 mm proved to be the slit dimension of choice which provides highest sensitivity.

This method is based on the difference in the R_f values of FCV ($R_f = 0.84 \pm 0.03$) and the degradation product ($R_f = 0.48 \pm 0.02$) as shown in Figure 11. A linear relationship was found to exist between the integrated area under the peak of the separated bands at the selected wavelength (304 nm) and the corresponding concentration of FCV in the range of (0.75–5.25 µg/band). The regression equation was computed and found to be

$$A = 2.2520\,C + 0.1240 \quad r = 0.9995 \quad \text{at 304 nm} \quad (6)$$

where A is the integrated area under the peak × 10^{-4} for FCV, C is the concentration of FCV in µg/band and r is the correlation coefficient.

The proposed method is valid for the determination of FCV in presence of up to 90% of its alkaline degradation product in different laboratory prepared mixtures as presented in Table 1, with mean percentage recovery 100.16 ± 0.91.

As the method could effectively separate the drug from its degradation product, it can be employed as a stability-indicating one.

High performance liquid chromatography

A simple isocratic high-performance liquid chromatographic method was developed for the determination of FCV in pure form and in pharmaceutical preparation.

To improve separation of peaks it was necessary to investigate the effect of different variables. Studying the optimum parameters for maximum separation was carried out as follows:

Stationary phase. Different types of stationary phase C_8 and C_{18} columns with different dimensions and particle size were tried (Agilent C_8 Zorbax, Agilent C_{18} Zorbax TM, Agilent C_8 Eclipse and Agilent C_{18} Eclipse columns), to get the best stationary-mobile phase match. It was clearly found that C_{18} Zorbax [(ODS), 10 µm (150 mm × 4.6 mm, i.d.)], Agilent (USA) gave the most suitable resolution for quantification of FCV and its degradation product.

Mobile phase. Different mobile phases at different pH values and varying organic modifiers, including acetonitrile and methanol, have been tested for optimizing the HPLC separation. The mobile

Table 3. Determination of FCV in Famclovir tablets by the proposed methods

	D^1 at321 nm		DD^1 method at 256 nm		Ferric hydroxamate method		TLC		HPLC	
Pharmaceutical dosage form	Found Mean ± RSD	Std Add Mean ± RSD	Found Mean ± RSD	Std Add Mean ± RSD	Found Mean ± RSD	Std Add Mean ± RSD	Found Mean ± RSD	Std Add Mean ± RSD	Found Mean ± RSD	Std Add Mean ± RSD
Famclovir tablets B.No. 0611292	98.89 ± 0.91	99.74 ± 1.19	100.19 ± 0.84	99.91 ± 0.89	99.32 ± 0.60	100.65 ± 0.89	99.74 ± 1.19	99.74 ± 1.19	98.56 ± 0.72	99.96 ± 0.81
Famclovir tablets B.No. 0611293	99.26 ± 0.88	99.71 ± 0.56	100.03 ± 0.71	100.26 ± 0.89	100.42 ± 0.54	100.72 ± 0.46	99.71 ± 0.56	99.98 ± 0.67	100.12 ± 0.68	99.50 ± 0.84

Std Add: Standard addition technique.

phase selection was based on peak parameters (symmetry and tailing), run time, ease of preparation, and cost. The mobile phase consisting of methanol–50 mM dipotassium hydrogen phosphate, pH was adjusted to 3.0 ± 0.1 using o-phosphoric acid (25 : 75, v/v) and flowing at 1.0 ml/min was found to be quite satisfactory for the good resolution and determination of the FCV in the presence of its degradation product. The decrease in the ratio of K2HPO4 or increase in the flow rate leads to bad resolution between peaks.

Detector wavelength. For the determination of the optimum HPLC-UV detector wavelength, the method was repeated using the same chromatographic conditions at different wavelengths (220–320 nm), where the optimum wavelength with ideal sensitivity and minimum noise was at 304 nm and was quite far from the cut-off of water and methanol. Upon applying the previously mentioned chromatographic conditions, well-resolved, sharp peaks of FCV and its degradation product appeared at retention times of 2.87 min. and 0.92 min in order. The total run time for a complete quantification of the drug and its degradation product was ~5 min. As Figure 12 shows, a typical chromatogram was obtained from the analysis of a laboratory prepared mixture of FCV and its degradation product by using the proposed method.

System suitability. System suitability parameters[26,27] were calculated under the optimized experimental conditions. The FCV and the degradation product could be eluted in the form of symmetrical peaks quite away from each other and the retention time values of the separated peaks together with other chromatographic parameters are shown in Table 2. The table describes the calculated resolution value (Rs) as well as selectivity factor (α), which ensures complete or 100% separation of the components under investigation. The tailing factor of the studied drug peak also revealed a linear isotherm peak elution without tailing.

Robustness. The robustness of the HPLC method was investigated by the analysis of samples under a variety of experimental conditions, such as small changes in the pH (3.0–3.5) and small changes in proportions of different components by up to ±0.5% mainly of the organic part of the mobile phase, plus the ionic strength of the phosphate salt component. The effect on retention time and peak parameters was studied. It was found that the method was robust when the column and the mobile phase ratio were varied. During these investigations the retention times were modified, however the areas and peaks symmetry were conserved.

This HPLC method has an advantage over the reported HPLC method,[6] being a stability-indicating method, while the reported HPLC is not because both the intact drug and its alkaline degradation product have the same retention time and their peaks are overlapped on each other.

The calibration graph was obtained by plotting the relative peak area ratio to the concentration of FCV (μg/ml). The linearity range was found to be 20–240 μg/ml using the following regression equation

$$A = 0.0181C - 0.0359 \quad r = 0.9996 \quad \text{at } 304 \text{ nm} \qquad (7)$$

where A is the peak area ratio, C is the concentration of FCV (μg/ml) and r is the correlation coefficient.

The proposed method is valid for the determination of FCV in presence of up to 90% of its alkaline degradation product in different laboratory prepared mixtures as presented in Table 1, with mean percentage recovery 99.92 ± 1.42.

Application to pharmaceutical preparation

The suggested methods are valid and applicable for the analysis of FCV in Famclovir® tablets. The validity of the proposed methods was assessed by applying the standard addition technique, which showed accurate results with no interference from excipients as shown in Table 3.

Method validation

Method validation was performed according to USP guidelines[28] for all the proposed methods as follows:

Range and linearity

The linearity of the method was evaluated by processing 6-point calibration curves on three different days. The calibration curves were constructed within concentration ranges that were selected on the basis of the anticipated drug concentration during the assay of the dosage form. A linear least-squares regression analysis was conducted to determine slope, intercept, and coefficient of determination to demonstrate linearity of the method. The goodness of fit in all cases was found to be >0.9966, indicating a functional linear relationship. The relevant slope values were statistically different from zero ($P < 0.05$), and though the intercepts of the calibration curves were significantly different from zero, they did not affect the accuracy of the method. The linear regression analysis data is summarized in Table 4.

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated respectively according to the current ICH guidelines[29,30] with a ratio of 3.3 and 10 standard deviations of the blank and the slope of the calibration line (Table 4).

Accuracy

To study the accuracy of the proposed methods, procedures under study of linearity were repeated three times for the determination of six different concentration of pure FCV. The accuracy expressed as percentage recoveries is shown in Table 4. Good accuracy of the developed methods was indicated by the results obtained.

Precision

The precision of the proposed method, expressed as RSD, was determined by the analysis of three different concentrations of pure FCV within the linearity range of FCV. The intraday precision was assessed from the results of three replicate analyses of pure FCV on a single day. The inter-day precision was determined from the same samples analyzed on three consecutive days. The results of intra-day and inter-day precisions are illustrated in Table 4.

Table 4. Assay parameters and validation sheet for the determination of FCV in pure powder form by the proposed methods

Parametes	D^1 method	DD^1 method	Ferric hydroxamate method	Densitometric method	HPLC method
Range	16–72 μg/mL	40–240 μg/mL	40–240 μg/mL	0.75–5.25 μg/band	20–240 μg/mL
LOD	1.44	3.90	2.22	0.018	0.60
LOQ	4.8	10.8	7.41	0.06	2.00
Slope	0.006	0.005	0.0058	2.2520	0.0181
Intercept	0.0789	0.0570	−0.0277	0.1240	−0.0359
Correlation coefficient	0.9998	0.9996	0.9998	0.9995	0.9996
Accuracy Mean ± S.D	99.49 ± 0.65	99.89 ± 0.75	99.94 ± 0.77	100.12 ± 1.15	99.57 ± 0.58
RSD%[a] *	0.920	0.989	1.512	1.625	0.743
RSD% [b] *	0.900	0.942	1.001	1.580	0.602

RSD%[a] * Inter-day and RSD% [b] * Intra-day of samples of concentrations (15, 30, 60 μg/.ml) for D^1, (40, 80, 160 μg/.ml) for DD^1, (40, 80, 160) for ferric hydroxamate method, (0.75, 1.5, 3 μg/band) for densitometric method and (40, 80, 160 μg/.ml) for HPLC method.

Table 5. Statistical comparison for the results obtained by the proposed methods and the reported method for the analysis of FCV

Parameter	D^1-Method At 321 nm	DD^1-Method At 256 nm	Ferric hydroxamate method method	TLC-densitometric method	HPLC method	** Reported method
Mean	99.65	100.27	99.72	100.65	99.88	99.97
S.D.	0.85	0.91	0.84	1.52	0.50	0.40
Variance	0.72	0.82	0.7	2.31	0.25	0.16
N	6	6	6	6	6	3
F-test	4.50(19.3)*	5.12(19.3)*	4.37(19.3)*	14.43(19.3)*	1.56(19.3)*	
Student's t-test	0.61(2.365)*	0.54(2.365)*	0.48(2.365)*	0.74(2	0.27(2.365)*	

* The values in the parenthesis are the corresponding theoretical *t*- and F-values at P = 0.05
** HPLC method, 150 mm × 4.6 mm i.d. C_{18} A ZORBAX column with a mobile phase composed of 50 mM monobasic phosphate buffer and methanol (50 : 50 v/v), adjusted to pH 3 with orthophosphoric acid.

Statistical analysis

Table 5 shows statistical comparison of the results obtained by the proposed methods and the reported HPLC method.[6] The calculated *t*- and F-values are less than the theoretical ones indicating that there is no significant difference between the proposed methods and the reported method[6] with respect to accuracy and precision.

One-way ANOVA was applied for the comparison of those methods, where there was no significant difference between the proposed methods and the reported method[6] (Table 6).

Table 6. Statistical comparison for the results obtained by the proposed methods and the reported method for the analysis of FCV using one-way ANOVA

Source	DoF	Sum of Squares	Mean Square	F Value	P Value
Model	5	1.57262222	0.314524444	0.37503	0.86177
Error	30	25.1602000	0.838673333		

At the 0.05 level, the population means are not significantly different.

Conclusion

The present work is concerned with the determination of FCV in the presence of its alkaline degradation product. In this paper, simple, sensitive and rapid methods are described for the determination of FCV in pure form or in pharmaceutical formulations.

Reviewing the literature in hand, no other spectrophotometric or TLC- densitometric methods concerned with the determination of FCV in the presence of its alkaline degradation product have been identified in other published methods.

These D^1 and DD^1 spectrophotometric methods are well-established techniques that are able to enhance the resolution of overlapping bands. These methods are simple, more convenient, less time-consuming and economic stability-indicating methods compared to other published methods.

Ferric hydroxamate method is specific for FCV in the presence of its alkaline degradation product, so it is a stability-indicating method. It can be recommended for routine analytical laboratories when sophisticated, expensive equipments are unavailable.

The advantage of TLC-densitometric and HPLC method is that several samples can be run simultaneously, thus lowering analysis time and cost per analysis and providing high sensitivity and selectivity.

The proposed HPLC method is simple and rapid (total run time ~5 min) for separation of FCV and its degradation product.

High values of correlation coefficients and small values of intercepts validated the linearity of the calibration graphs and the obedience to Beer's Law. The RSD values, the slopes and the intercepts of the calibration graphs indicated the high reproducibility of the proposed methods.

From the results obtained, it is concluded that the suggested methods show high sensitivity, accuracy, reproducibility and specificity and can be used as stability indicating methods. Moreover, these methods are simple and inexpensive, permitting their application in quality control laboratories.

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