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Development and validation of a stability-indicating RP-LC method for famciclovir

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ABSTRACT

A novel stability-indicating gradient reverse phase liquid chromatographic (RP-LC) method was developed for the determination of purity of famciclovir (FCV) in presence of its impurities and degradation products. The method was developed using Inertsil ODS 3 V (250 × 4.6 mm, 5 μm) column with mobile phase containing a gradient mixture of solvent A and B. 0.01 M potassium dihydrogen orthophosphate buffer, pH adjusted to 6.0 with 1% potassium hydroxide was used as buffer. Buffer and methanol in 80:20 (v/v) ratio was used as solvent A and buffer and methanol in 20:80 (v/v) ratio was used as solvent B. The gradient program (T/%B) was set as 0/5, 15/30, 25/50, 45/60, 55/5 and 60/5. The eluted compounds were monitored at 215 nm. FCV was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. FCV was found to degrade significantly in oxidative, acid and base degradation conditions and mildly in hydrolytic degradation conditions and stable in thermal and photolytic degradation conditions. The degradation products were well resolved from main peak and its impurities thus proved the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantitation, precision, linearity, accuracy, robustness and system suitability. This method is also suitable for the assay of famciclovir which ranged from 99.9% to 100.2%.

1. Introduction

Stability-indicating method can selectively analyze the parent constituent (active pharmaceutical ingredient, API) from the pharmaceutical product. It is to be developed either to determine only the parent constituent or only the impurity or degradation product or to separate and determine the parent compound in presence of its impurities and degradation products [1]. Regulatory guidance in ICH Q1AR2 [2], Q3BR2 [3], Q6A [4] and FDA 21 CFR section 211 [5] insists for the validated stability-indicating purity and assay methods [1]. Forced degradation conditions, stress agent concentration and times of stress are to be established in such a way that they effect degradation, preferably 10–20% of the parent constituent. The discovery of such conditions is based on trial and error methods.

Famciclovir (FCV), chemically known as 2-[2-(2-amino-9*H*-purine-9-yl)ethyl]-1,3-propane diol diacetate [6] is a guanine analogue antiviral drug used for the treatment of various herpesvirus infections. It is a prodrug of penciclovir with improved oral bioavailability. Three RP-LC methods were found in literature for the determination of famciclovir in pharmaceutical formulations [7–9], but forced degradation details were not presented in these articles. The reported assay method [10] was also out of scope because it did not determine the impurities. Hence, in continuation to our research towards the determination of impurities in APIs [11,12], we have developed a stability-indicating RP-LC method that can separate and quantitate FCV, its process related impurities and degradation products (Fig. 1). The method was validated as per International Conference on Harmonization (ICH) guidelines [13].

2. Experimental

2.1. Chemicals and reagents

FCV (99.8%) and its six impurities, viz. Imp-1 (97.5%), Imp-2 (99.8%), Imp-3 (97.8%), Imp-4 (96.8%), Imp-5 (98.2%) and Imp-6 (97.6%) were obtained from our R & D division. Imp-1 and Imp-4 are the forced degradation products, Imp-2 is major metabolite and Imp-3, Imp-5 and Imp-6 are the process related impurities. Potassium dihydrogen orthophosphate monohydrare, potassium hydroxide, methanol, hydrochloric acid, sodium hydrox-

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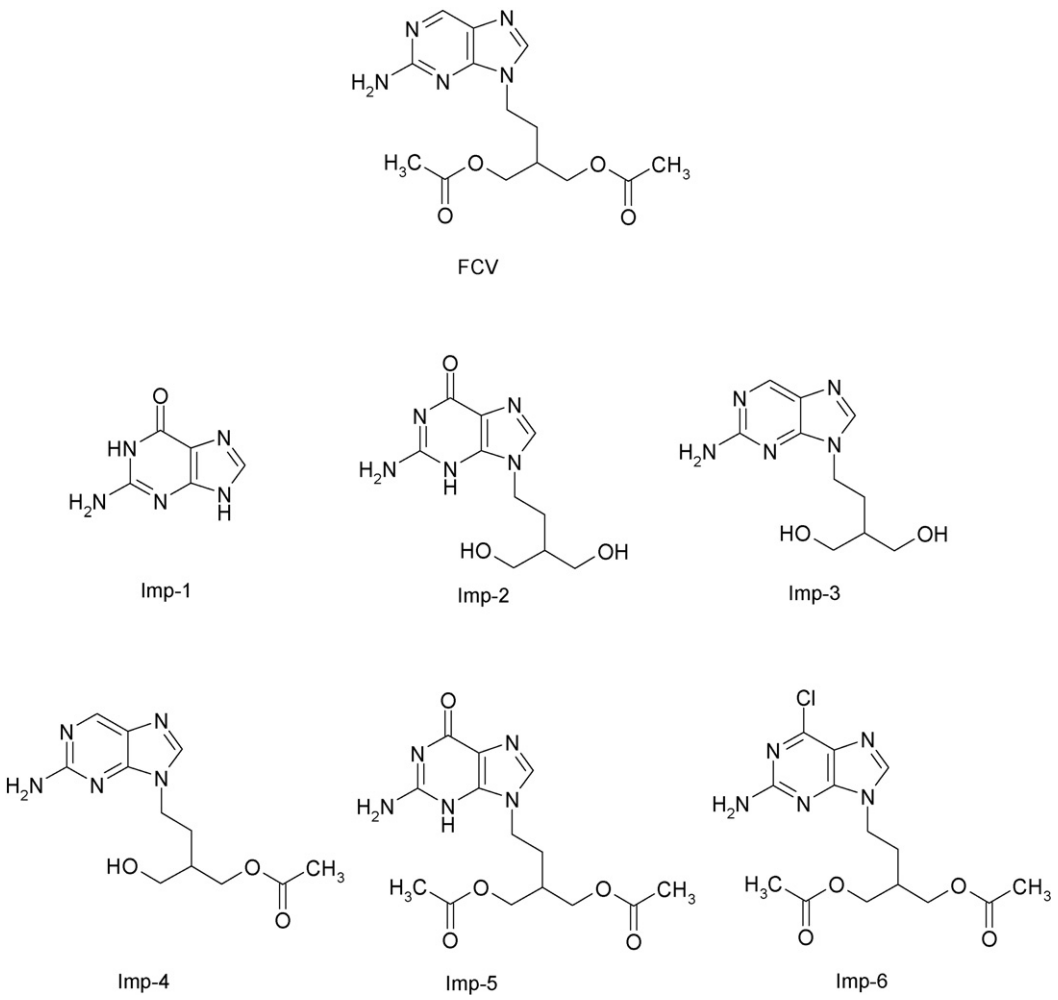

Fig. 1. Structures of FCV and its six impurities.

ide and hydrogen peroxide (30%) were obtained from Merck, India. All the solutions were prepared in Milli Q water (Millipore, USA).

2.2. HPLC conditions

Waters Alliance e2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/vis detector or 2998 PDA detector (for specificity and forced degradation studies) with empower2 software was used for the analysis. Inertsil ODS 3 V column (250 × 4.6 mm, 5 μm, GL Sciences, Japan) and a gradient mixture of solvent A and B were used as stationary and mobile phases, respectively. 0.01 M Potassium dihydrogen orthophosphate buffer, pH adjusted to 6.0 with 1% potassium hydroxide was used as buffer. Buffer and methanol in 80:20 (v/v) ratio was used as solvent A and buffer and methanol in 20:80 (v/v) ratio was used as solvent B. The gradient program (T/%B) was set as 0/5, 15/30, 25/50, 45/60, 55/5 and 60/5. Solvent A was used as diluent. 0.8 ml/min flow rate was maintained. The eluted compounds were monitored at 215 nm. The column oven and auto sampler temperatures were maintained at 27 °C and 5 °C, respectively. An injection volume of 10 μl was used.

2.3. LC–MS/MS conditions

LC–MS/MS system (Waters 2695 Alliance liquid chromatograph coupled with quattromicromass spectrometer with MassLynx software, Waters Corporation, Milford, USA) was used for the identification of Impurities formed during forced degradation studies. Inertsil ODS-3 V (150 × 4.6 mm, 5 μm, GL Sciences, Japan) column and a gradient mixture of solvent A and B were used as stationary and mobile phases, respectively. 0.01 M ammonium formate was used as buffer. Buffer and methanol in 80:20 (v/v) ratio was used as solvent A and buffer and methanol in 20:80 (v/v) ratio was used as solvent B. The gradient program (T/%B) was set as 0/5, 15/30, 25/50, 45/60, 55/5 and 60/5. Solvent A was used as diluent. The flow rate was 0.4 ml/min. The analysis was performed in positive electro spray positive ionization mode. Capillary and cone voltages are 3.5 kV and 25 V, respectively. Source and dissolvation temperatures are 120 °C and 350 °C, respectively. Dissolvation gas flow is 650 l/h.

2.4. Preparation of stock and standard solutions

Solvent A was used as diluent. 1 and 0.1 mg/ml stock solutions of FCV were prepared in diluent for purity and assay methods, respectively. 0.15% of six impurities blend with respect to 1 mg/ml FCV was prepared in diluent.

2.5. Forced degradation conditions

In order to determine whether the method is stability-indicating, forced degradation studies were conducted on FCV API powder. The analysis was carried out by HPLC with a PDA detector.

10 μl of each of forced degradation samples were injected at regular intervals and the final stress conditions were established in such a way that 10–20% degradation of FCV was occurred. LC–MS/MS system was used for the identification of unknown compounds formed during forced degradation studies.

2.5.1. Oxidative degradation

100 mg of FCV sample was taken into a 100 ml round bottom flask, 10 ml of 10% hydrogen peroxide solution was added, contents were mixed well and kept for constant stirring for 5 h at room temperature. 1.0 ml of this solution was diluted to 10 ml with diluent.

2.5.2. Acid degradation

100 mg of FCV sample was taken into a 100 ml round bottom flask, 10 ml of 0.1 M hydrochloric acid solution was added, contents were mixed well and kept for constant stirring for 3 h at room temperature. 1.0 ml of this solution was taken in 10 ml volumetric flask and neutralized with 1.0 ml of 0.1 M sodium hydroxide and then diluted to 10 ml with diluent.

2.5.3. Base degradation

100 mg of FCV sample was taken into a 100 ml round bottom flask, 10 ml of 0.005 M sodium hydroxide solution was added and the contents were mixed well. 1.0 ml of this solution was taken in 10 ml volumetric flask and neutralized with 1.0 ml of 0.005 M hydrochloric acid and then diluted to 10 ml with diluent.

2.5.4. Hydrolytic degradation

100 mg of FCV sample was taken into a 100 ml round bottom flask, 10 ml of Milli Q water was added, the contents were mixed well and kept for constant stirring for 8 h at 80 °C. 1.0 ml of this solution was diluted to 10 ml with diluent.

2.5.5. Thermal degradation

1.0 g of FCV sample was taken in to a petri dish and kept in oven at 80 °C for 7 days. 10.0 mg of this sample was taken in to a 10 ml volumetric flask, dissolved in diluent and diluted to volume with diluent.

2.5.6. Photolytic degradation

1 g of FCV sample was taken in to a petri dish and kept in photo stability chamber/200 W h/m^2 in UV light and 1.2 million lx h in visible light for 7 days. 10.0 mg of this sample was taken in to a 10 ml volumetric flask, dissolved in diluent and diluted to volume with diluent.

3. Results and discussion

3.1. HPLC method development

HPLC method development for the separation between FCV and its six impurities was initiated with the literature method [10]. In this method, Imp-1, Imp-2 and Imp-3 are co-eluting at the same retention time. Hence, the affect of mobile phase pH is studied at 3.0, 4.5 and 6.0. These buffers were prepared by taking 0.01 M potassium dihydrogen orthophosphate buffer and the pH was adjusted with orthophosphoric acid. Buffer with pH 6.0 was found to be suitable as all the Impurities are well separated with better resolutions. Since Imp-1 is eluting too early and Imp-6 is eluting at too longer retention time, the method is modified to gradient. However, blank interference for Imp-3 and Imp-5 was observed. Hence, acetonitrile is replaced with methanol. Different diluents were also tried for this purpose and a neat base line was achieved with a gradient mixture of solvent A (Buffer and methanol in 80:20 v/v ratio) and B (Buffer and methanol in 20:80 v/v ratio) as mobile phase components and using solvent A as diluent. Several gradient programs were tried and a gradient program of 0/5, 15/30, 25/50, 45/60, 55/5 and 60/5 (T/%B) was found to be optimal as all the process Impurities of FCV are eluted at adequate retention times. However, peak shapes are not good for Imp-4 and Imp-6. Hence, different C18 columns were tried to improve the peak shapes and responses of FCV and its impurities. Inertsil ODS 3V (250 × 4.6 mm, 5 μm) column was found to be suitable for the intended use. 215 nm wavelength is selected as FCV and its six impurities are having maximum UV absorbance at this wave length. 10 μl injection volume, 0.8 ml/min flow rate, 27 °C temperature were found to be ideal for a good chromatographic performance. The chromatographic performance data is presented in Table 1. A typical chromatogram of FCV spiked with 0.15 % of its six impurities is shown in Fig. 2.

Table 1
Chromatographic performance data.

Compound	RT (Min)	RRT*	Resolution#	Tailing factor
Imp-1	4.7	0.23	–	1.42
Imp-2	5.6	0.28	4.5	1.36
Imp-3	7.3	0.36	7.8	1.15
Imp-4	13.1	0.65	23.9	1.10
Imp-5	17.3	0.86	7.4	0.77
FCV	20.0	1.00	6.6	1.13
Imp-6	25.6	1.28	15.4	0.99

* Relative retention times (RRT) were calculated against the retention time (RT) of FCV.
# Resolutions were calculated between two adjacent peaks.

3.2. Method validation

The proposed method was validated as per ICH guidelines [13].

3.2.1. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. The specificity of the developed LC method was checked in the presence of its impurities and forced degradation products. For this purpose, all the stressed samples of FCV were spiked with its six process impurities (0.15% with respect to FCV concentration). All the impurities and degradants are well resolved from one another and FCV peak indicating the specificity of the proposed method to quantify FCV and its six impurities.

3.2.2. Limit of detection limit and quantitation

Initially, 0.15% of FCV impurities blend with respect to 1 mg/ml FCV was injected. This solution was further diluted to achieve the signal-to-noise (S/N) ratio of 3:1 and 10:1 for determining limit of detection (LOD) and limit of quantitation (LOQ), respectively. The S/N ratio was for FCV impurities were calculated using Waters Empower2 software. The determined limit of detection and limit of quantitation values for the six impurities of FCV were reported in Table 2.

3.2.3. Precision

The precision of the method was checked by injecting six individual preparations of FCV spiked with 0.15% of its six impurities (0.15% of impurities with respect to 1 mg/ml FCV). %RSD of area for each impurity was calculated. LOQ precision also determined by injecting six individual preparations of FCV spiked at LOQ level of its six impurities (with respect to 1 mg/ml FCV). The intermediate precision of the method was also verified on six different days in the same laboratory using the specification and LOQ spiked FCV solutions prepared as above. Columns with different packing lot particles were used during this study and the results were found to be identical. Assay method precision was evaluated by carrying out six independent assays of test sample of FCV at 0.1 mg/ml level

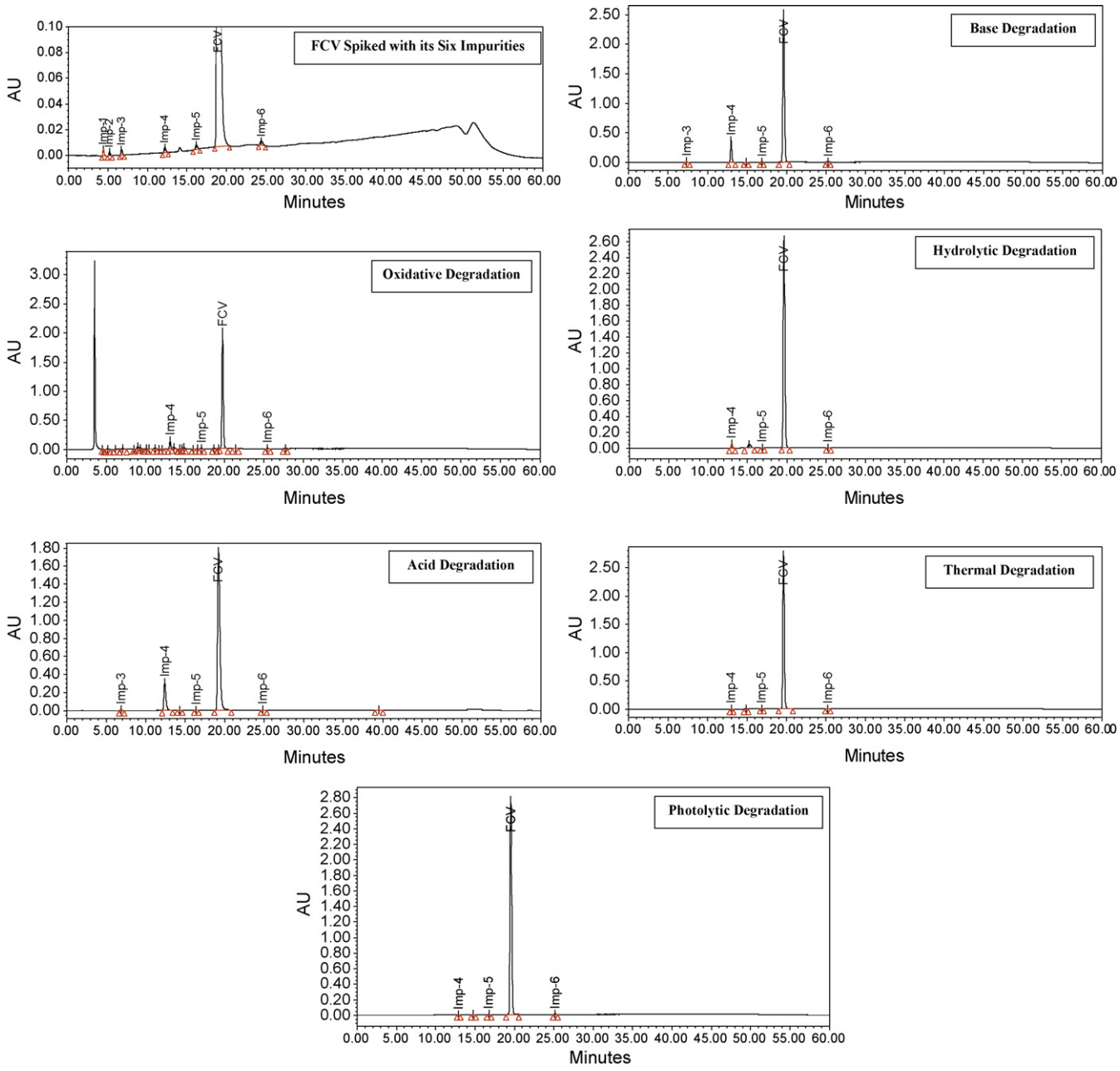

Fig. 2. Typical chromatograms of FCV spiked with its six impurities and its forced degradation samples.

Table 2
Regression and precision data.

Parameter	FCV	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
LOD (%)	NA	0.015	0.010	0.013	0.016	0.014	0.011
LOQ (%)	NA	0.045	0.031	0.039	0.046	0.043	0.032
Regression equation (y)							
Slope (b)	2567.1	1549.2	1276.9	3759.1	1297.3	2387.1	4265.2
Intercept (a)	201.3	305.2	321.2	78.2	222.5	176.2	122.5
Correlation coefficient	0.9998	0.9997	0.9999	0.9998	0.9998	0.9998	0.9999
Precision (%RSD)[#]	0.72	0.54	0.45	0.89	0.36	0.76	1.02
Intermediate precision (%RSD)[#]	1.53	1.65	1.88	1.87	1.67	1.82	1.76

NA: Not applicable for assay as per ICH guidelines. Linearity range is LOQ – 200% with respect to 1 mg/ml of FCV for impurities. Linearity range is 50–150% with respect to 0.1 mg/ml FCV for assay.

[#] Six determinations using LOQ solution for impurities and 0.1 mg/ml for assay.

Table 3
Evaluation of accuracy.

Amount spiked*	%Recovery#					
	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
LOQ	99.1 ± 0.22	98.7 ± 0.22	99.2 ± 0.34	99.6 ± 0.23	99.1 ± 0.23	98.8 ± 0.25
100%	99.4 ± 0.23	99.5 ± 0.34	99.3 ± 0.62	99.8 ± 0.32	99.2 ± 0.32	99.3 ± 0.41
150%	99.5 ± 0.34	98.6 ± 0.43	99.1 ± 0.18	99.1 ± 0.54	99.1 ± 0.31	99.1 ± 0.12

* Amount of six impurities spiked with respect to 0.15% specification level individually to 1 mg/ml of FCV.
# Mean ± %RSD for three determinations.

Table 4
Summary of forced degradation studies.

Stress conditions	%Degradants formed								%Assay	Mass balance(%)
	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	SMU	Total impurities		
Unstressed	ND	ND	ND	0.05	0.04	0.05	0.13	0.20	–	–
Oxidative degradation	ND	ND	ND	5.20	0.39	0.11	2.60	13.60	86.0	99.6
Acid degradation	ND	ND	0.48	12.90	0.07	0.07	0.10	13.80	86.0	99.8
Base degradation	ND	ND	0.46	10.70	0.08	0.09	0.10	11.70	87.5	99.2
Hydrolytic degradation	ND	ND	ND	1.76	0.10	0.10	3.05	5.10	94.2	99.3
Thermal degradation	ND	ND	ND	0.05	0.04	0.05	ND	0.20	99.5	99.7
Photolytic degradation	ND	ND	ND	0.06	0.04	0.05	0.04	0.20	99.6	99.8

SMU: Single maximum unknown; ND: Not detected.

against qualified reference standard. The intermediate precision of the assay method was evaluated by different analysts. The %RSD values are presented in Table 2.

3.2.4. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. Linearity test solutions for impurities were prepared individually at six concentration levels (each triplicate) in the range of LOQ to 200% of the specification level, viz. 0.15%. Linearity test solutions for FCV assay were prepared from stock solution at five concentration levels from 50% to 150% of assay analyte concentration (0.1 mg/ml of FCV). The peak area versus concentration data was performed by least-squares linear regression analysis. The correlation coefficient of the respective calibration curve was calculated (Table 2).

3.2.5. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. Standard addition and recovery experiments were conducted to determine accuracy of the impurities of FCV for their quantification. The study was carried out in triplicate at LOQ, 100% and 150% with respect to specification level, viz. 0.15%. The percentage recoveries are presented in Table 3. The accuracy of the FCV assay was evaluated in triplicate at three concentration levels, viz. 50%, 100% and 150% with respect to 0.1 mg/ml of FCV test concentration. The assay of famciclovir ranged from 99.9% to 100.2%.

3.2.6. Robustness

The robustness was illustrated by getting the resolution between any two compounds to be greater than 2.0, when mobile phase flow rate (±0.2 ml/min), pH (±0.2), organic solvent ratio (±5%) and column temperature (±2 °C) were deliberately varied.

3.2.7. Solution stability

The solution stability of FCV and its six impurities in diluent was determined by leaving 0.15% spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h and measuring the amounts of the six compounds for every 6 h. The solution stability of FCV assay (in diluent) was determined by leaving 0.1 mg/ml FCV solution in tightly capped volumetric flasks at room temperature for 48 h during which they were assayed at 6 h intervals and comparing the results with those obtained from freshly prepared solution. The mobile phase was prepared at the beginning of the study period and not changed during the experiment. The %RSD values for solution stability experiments were calculated and found to be 1.22 and 1.35 for purity and assay methods, respectively. All the samples were found to be stable up to 48 h.

3.2.8. System suitability

The resolution between Imp-2 and Imp-1 should not be less than 3.0, when a mixture containing 0.15% each of these two impurities with respect to 1 mg/ml of FCV was injected.

3.3. Results of forced degradation studies

Significant degradation of FCV was observed in oxidative, acid and base degradation conditions leading to the formation of Imp-4. This was confirmed by co-injecting Imp-4 to these degraded samples and LC–MS analysis. Mild degradation of FCV was observed in hydrolytic degradation condition. FCV was found to be stable under thermal and photolytic degradation conditions (Fig. 2). Photodiode array detector was employed to check and ensure the homogeneity and purity of FCV peak in all the stressed sample solutions. Assay studies were carried out for stress samples against FCV qualified working standard and the mass balance (%assay + %sum of all impurities + %sum of all degradants) results are presented in Table 4. The purity and assay of FCV was unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the developed method.

4. Conclusions

A stability-indicating HPLC method has been developed and validated for the determination of FCV and its six impurities. The behavior of FCV under various stress conditions was studied. Since the method is able to separate the FCV from its impurities and degradation products, it can be used for checking the quality of FCV in its bulk and stability samples.

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