REDVET - Revista electrónica de Veterinaria
ISSN 1695-7504
Vol 25, No. 1 (2024)
http://www.veterinaria.org
Article Received: 27 June 2024 Revised: 30 July 2024 Accepted: 28 August 2024

Exploring Densitometric Coupled With Spectrophotometric Analytical Approaches For Nucleotide Active Analogue For Retinitis Infection: In-Situ Measurement In Injection

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ABSTRACT:
For the analysis of cidofovir in bulk and in custom injection formulations, new, affordable, quick, and effective HPTLC and UV-spectrophotometry area under curve (UV-AUC) procedures were created and validated. The analysis was performed using (10 x 10 cm) aluminium sheets precoated with silica gel 60-F_{254} (E. Merck) as the stationary phase and dichloromethane, methanol, and triethylamine (3:2:0.5 v/v/v) as the mobile phase. Cidofovir was quantitated by NP-HPTLC using UV detection at 273 nm. Quantitation using the HPTLC technique was done at concentrations between 500 - 3000 ng/band. Cidofovir produced a compact and distinct band with a retardation factor (R_f) of 0.45 ±0.02 using the HPTLC technique. The calibration of the HPTLC method using linear regression analysis showed a satisfactory linear relationship with a regression coefficient of $r^2 = 0.995$. SLS (0.03 w/v) was used as the solvent in the development of the UV-AUC method, and the area was calculated at a wavelength between 266.40 and 278.80 nm. The UV-AUC analysis was shown to have a $r^2 = 0.999$ correlation coefficient. In a concentration range of 5–30 µg/mL, the newly developed UV-AUC technique showed a fine linear relationship for cidofovir. The designed methodologies were validated for precision, robustness, ruggedness, accuracy, and sensitivity in accordance with guidelines published by the International Conference on Harmonization (ICH). The developed procedures can be successfully applied to the study of cidofovir in *in-house* injection and bulk form because statistical analysis revealed them to be precise, robust, sensitive, and accurate.

Keywords: Cidofovir; HPTLC; UV-AUC

1. INTRODUCTION

Cytomegalovirus (CMV) retinitis in AIDS patients is treated with the antiviral medication cidofovir. Cidofovir is 1 - ((3 hydroxy - 2 phosphonyl methoxy) propyl) cytosine (CIDO). For the treatment of cytomegalovirus (CMV) retinitis in AIDS patients, an injectable antiviral medication called cidofovir is administered. Through the specific suppression of viral DNA synthesis, it prevents CMV replication. The FDA gave their clearance to it in 1996. [1]

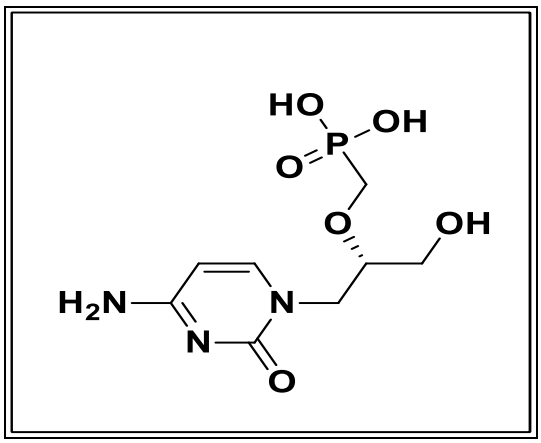

Figure 1. Chemical Structure of CIDO

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ISSN 1695-7504
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A review of the literature found that there aren't many analytical techniques available for detecting cidofovir in biological fluids, pharmaceutical formulations, and bulk such as HPLC determination of cidofovir in samples from both skin layers and percutaneous penetration [3]. RP-HPLC method for determination of Cidofovir in medicinal form analysis: development and validation [4]. Isolation and identification of a metabolite of cidofovir from rat kidney also reported [5]. LC-MS/MS quantification of cidofovir was performed in human serum for paediatric use [6]. In a mouse model infected with MuPyV, a quantitative LC-MS/MS approach was used to measure the levels of tissue brincidofovir and cidofovir diphosphate [7]. High-performance liquid chromatography-tandem mass spectrometry determination of cidofovir in human plasma following low dosage medication administration [8].

Hydrotropy is defined as increasing the solubility of different substances in water, and it could be possible by adding large quantities of second solvent (hydrotropic agents) [9]. Specific hydrotropic solutions have been used to solubilize the poorly water soluble products. Sodium benzoate, niacinamide, sodium lauryl sulphate, sodium salicylate, sodium acetate, sodium citrate, and urea were used to improve the aqueous solubility of many poorly water-soluble drugs [10]. The number of drugs was analyzed using hydrotropy approach including Tolvaptan, Fenticonazole, Risperidone, and Ticagrelor[11-14]. The Area Under Curve (AUC) technique applies where no sharp spectra is obtained or broad spectrum is obtained. This requires measuring the combined AUC value between the two chosen wavelengths λ1 and λ2. The objective of this investigation is to develop an easy, rapid, precise, reproducible, eco-friendly and economical method for determination of fenticonazole nitrate using UV-spectrophotometry by analyzing absorbance and Area Under Curve (AUC) techniques [15-19].

In order to support regular quality control of cidofovir in bulk and *in-house* injection formulation, the proposed inquiry was designed to provide the construction of a novel; simple, efficient, rapid, and cheap analysis of cidofovir with application of NP-HPTLC and UV-AUC analyses in accordance with ICH guidelines [20].

2. EXPERIMENTAL

2.1 Drug and chemicals

Sodium hydroxide, methanol, dichloromethane, triethylamine and were used of HPLC grade procured from Merck Ltd., Mumbai and used without further purification. Cidofovir was gifted from Emcure Laboratories PVT. LTD Pune, India. Analytical quality chemicals and reagents were bought from Mumbai's Merck Fine Chemicals.

2.2 Equipments and experimental conditions

An ultrasonicator, manufactured by ENERTECH Electronics Pvt. Ltd., a Camag TLC system (Muttenz, Switzerland), a Hamilton syringe (100 µL), a Camag TLC scanner 3, Camag winCATS software (version 1.3.0), a Camag dual trough chamber (20 X10 cm), and a Camag TLC scanner 3 were utilized throughout the experiment. On silica gel 60-F254 (20X10 cm) HPTLC plates with an aluminium frame and a 200 lm thickness, chromatographic analysis was carried out (E. Merck, Mumbai, India). The HPTLC plates were cleaned in advance and dried in an oven at 100^0 C. A Camag TLC Scanner 3 (Camag, Muttenz, Switzerland) equipped with winCATS software was used for densitometric detection. The Linomat 5 (Camag) applicator was used to apply drug standards and samples to the HPTLC plates while nitrogen gas was circulating. 10 µL samples were spotted 6 mm from the plate's edge. In a twin trough glass chamber measuring 20 by 10 cm from Camag in Muttenz, Switzerland, the plates were developed. The mobile phase had a 10 mL volume. Prior to usage, mobile phase components were combined, and the development chamber was allowed to be saturated with mobile phase vapours for 20 minutes at room temperature (25^0 C). The plate was developed using the ascending process to a migration distance of 8 cm, and then it was dried with an air dryer. The slit size was preserved at 6 mm X 0.45 mm. (micro), and 20 mm was used as the scanning speed. A deuterium lamp exhibiting a continuous UV spectrum between 800-200 was used for densitometric scanning in the absorbance-reflectance mode at 260 nm.

2.2.2 For UV-AUC analysis

All spectroscopic measurements were carried out with a UV-Visible spectrophotometer (2450 Shimadzu, software UV Probe 2.21) using a pair of 10 mm identical quartz cell pairs. 0.03 M SLS (0.03 w/v) was utilised as a hydrotropic agent and wetting agent to increase the solubility of cidofovir and served as the solvent for the UV spectrophotometric measurement of cidofovir. We were able to make the medication entirely soluble in double-distilled water due to SLS's surfactant properties. The wetting, spreading, and surfactant properties of SLS are widely recognised, and it is frequently employed to help pharmaceuticals dissolve.

2.3. Preparation of standards:

2.3.1. For HPTLC analysis

A stock standard solution of 1000 µg/mL of cidofovir was obtained by weighing 10 mg of cidofovir with the assistance of a SHIMADZU AUX-120 analytical balance, diluting it in a 10 mL volumetric flask containing water (pH 8.0), and then sonicating it for 10 minutes with an ultrasonicator manufactured by ENERTECH Electronics Pvt. Ltd. in India. A appropriate volume of 0.5–3.0 mL of cidofovir was transferred from stock solution into a series of 10.0 mL volumetric flasks, and the volume was filled off again with water (pH adjusted to 8.0 using Sodium hydroxide solution). A volume

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of 10 µL was added to a TLC plate from each volumetric flask to obtain a range of concentrations that ranged from 500 to 3000 ng/band.

2.3.2. For UV-AUC analysis

To dissolve cidofovir in water and establish the drug's spectrum properties, 0.03M SLS was used as the solvent. SLS is employed as a hydrotropic agent to improve cidofovir's solubility in water. As stated in Section 2.3.1, a stock standard solution of cidofovir was created by precisely weighing 10 mg of the drug. The volume was raised up to the required level very slowly by adding 0.03M SLS to achieve a final concentration of 100 lg/mL for the standard solution used during UV-AUC analysis. Sonication in an ultrasonicator for 15 minutes was employed to remove any foam that may have been created. To avoid the development of foam and any errors in the analysis that may result from foam and entrapped air, care was taken and dilutions were made with very slow addition of 0.03%w/v. SLS. Following the proper dilutions, 10 µg/mL cidofovir was scanned between 400 - 200 nm in the UV area.

2.3.3. Samples preparation

A 10.0 mL volumetric flask was then filled with the precisely weighed 10 mg Cidofovir standard. It was dissolved in water (pH 8.0), and the volume was built up to the specified level using the same solvent to produce the desired concentration, or 1000 g/mL

3 RESULTS AND DISCUSSION

3.1 Mobile Phase Optimization.

Drug found effectively soluble in water (pH adjusted to 8.0 using Sodium hydroxide solution) and it was finally selected as solvent of choice for studies. Initially, toluene was tried Toluene: Methanol was next tested in a variety of ratios based on drug polarity, these important analyses revealed drug spot. A mobile phase made up of dichloromethane, methanol, and triethylamine (3:2:0.5 v/v/v) was ultimately chosen. The peak for cidofovir was discovered to be uniform, acute, and well defined with an Rf value of 0.45.

3.2 Linearity and calibration curve.

An adequate volume of 0.5-3.0 mL of cidofovir was transferred from the stock solution and then a series of 10.0 ml volumetric flasks were filled to the proper level with water (pH adjusted to 8.0 using Sodium hydroxide solution). 10 ml from each volumetric flask were transferred to a TLC plate to provide a range of concentrations from 500 to 3000 ng/band. The plate was created and scanned in accordance with the instructions under the appropriate chromatographic conditions. Plotting the peak area vs drug concentration for each band resulted in the development of a calibration curve. y = 10.177x + 311.51 are calibration equations with regression coefficients (r2 = 0.995), which are often regarded as evidence of a perfect fit.

By converting stock standard solutions containing 100 µg/mL of cidofovir into working standards that contain 0.5–3.0 µg/mL of cidofovir and scanning in the 400–200 nm UV range, the UV-Zero order and UV-AUC technique was confirmed for linearity. At 273 nm, cidofovir demonstrated maximum absorption (max). The area under curve (AUC) in Figure 3 was measured at wavelengths of 266.4 and 278.8 nm. The range of 5 to 30 µg/mL was found to correspond to a linear calibration curve for AUC versus concentration. It was discovered that the UV-AUC linear regression equation was y =0.0174x + 0.1629 with a regression coefficient (r2 = 0.999).

3.3 Precision

When an analytical method is used repeatedly to several samplings of a homogenous sample, precision is the degree of consistency between individual test findings. Utilizing the intra-day and inter-day precisions of HPTLC and UV-AUC investigations were assessed using linear regression data from the calibration graph and zileuton concentration levels of 500, 1000, and 1500 ng/band for HPTLC analysis and 5, 10, and 15 µg/mL for UV-Zero order and UV-AUC calculation, respectively. Utilizing % relative standard deviation, the established HPTLC, UV-Zero order, and UV-AUC procedures are tested for precision (percent RSD). It was established that the% RSD was within acceptable boundaries.

Table no:1 Precision studies for Cidofovir for HPTLC and UV- Zero order UV-AUC analyses.

Analysis		Intra-day		Inter-day	
	Concentration (ng/band)	Amount found (ng/band)	% RSD	Amount found (ng/band)	% RSD
HPTLC	1000	992.8	0.69	997.65	0.98
	1500	1491.2	0.57	1475.9	1.17
	2000	1978.65	0.43	1987.5	1.05
UV-AUC	10	9.999	0.56	90.890	0.89
	15	14.935	0.66	14.686	0.42
	20	19.884	0.73	19.188	0.70

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3.4 Limit of Detection and Limit of Quantification

In accordance with the International Conference for Harmonisation guidelines Q2(R1) The responses' absolute deviation and the slopes of the resulting calibration curves (n = 3) were used to determine the detection limits (LOD) and limit of quantification (LOQ) for both of the methods mentioned. (ICH, 1997; 2005 amendment incorporated). During the validation of the HPTLC method, cidofovir solutions of 500, 1000, 1500, 2000, 2500, and 3000 ng/band were applied to HPTLC plates for the evaluation of LOD and LOQ. For the UV-Zero order and UV-AUC investigation, the linearity range of 5, 10, 15, 20, 25 and 30 µg/ mL was employed to calculate LOD and LOQ.

The formulas LOD = 3.3 N/B and LOQ = 10 N/B were used to calculate the LOD and LOQ. N is the noise standard deviation of the drug's peak areas (n=3), and B denotes the slope of the corresponding calibration curve. The smallest concentrations at which an analyte may be accurately measured and identified using HPTLC, UV-Zero order, and UV-AUC analysis, respectively, were computed and found to be 49.87 ng and 166.23 ng, 0.46 µg and 1.53 µg, and 0.38 µg and 1.26µg, respectively.

3.5 Robustness

Six duplicates were performed with a concentration of 1500 ng/band to examine robustness. The effects of four parameters—mobile phase composition, volume, development distance, and saturation duration—were investigated in this work. The impact of a slight modification to the mobile phase's make-up on the results were investigated. Different dichloromethane, methanol, and triethylamine ratios (2.5:2.5:0.5, 1.5:3.5:0.5, v/v) were used to generate chromatograms. Changes were made to the development distance (8 0.5 cm, i.e. 7, 8, and 7.5 cm), the saturation time (20 ±5 min, i.e. 10, 15, and 20), and the mobile phase concentration (10 ±1 mL, i.e. 9, 11, and 12 mL. The intervals between applying Cidofovir to the plate and its development, as well as between its development and scanning, were altered (10, 20, or 30 min)

Table 2 Robustness for HPTLC analysis of Cidofovir [n= 3].

Parameters	% RSD
Mobile phase composition	
Dichloromethane: methanol: triethylamine (2.5:2.5:0.5 *v/v/v*)	1.26
Dichloromethane: methanol: triethylamine (1.5:3.5:0.5 *v/v/v*)	0.75
Mobile phase volume [mL]	
11	0.86
9	0.77
Development distance [cm]	
7	0.92
7.5	1.32
8	1.01
Duration of saturation [min.]	
10	1.05
15	1.11
20	0.71

3.6 Accuracy

By conducting the recovery study at three levels, the accuracy of the approach was assessed. Three different concentrations of the standard drug; 80%, 100%, and 120% of the drug were added to the pre-analyzed, known concentration of the drug standard Cidofovir solution to conduct recovery studies. Table 3 shows good recovery rates ranging from 98.63 % to 99.53 % for HPTLC, 99.05 % to 99.12 % for UV-Zero order, and 99.06 % to 99.06 % for UV-AUC analysis of the drug at varied additional concentrations.

Table 3 Recovery studies for Cidofovir for HPTLC, UV- Zero order and UV-AUC analyses.

Drug	Initial amount applied [ng]	Amount over spotted [ng]	% Recovered	% RSD
HPTLC		800	98.62	0.87
	1000	1000	99.30	0.54
		1200	99.50	0.77
		80	98.62	0.98
UV-AUC	10	100	99.06	1.58
		120	99.20	0.65

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3.7 Ruggedness

While UV-Zero order and UV-AUC were determined with a drug concentration of 15 µg/mL, the HPTLC method's robustness was evaluated with a concentration of 1500 ng/band. When the same data was analysed by two independent analysts under the same experimental and environmental settings, the processes were confirmed to be reliable.

Parameters	HPTLC	UV- AUC
Linearity range (µg/mL)	500 - 3000	5-30
Correlation coefficient	0.9995	0.9992
Analysis of Bulk material (% amount found)	98.96	100.93
Analysis of *in-house* injection (% amount found)	99.32	99.80
Ruggedness [% amount found ± SD]		
Analyst-I	99.28 ± 0.57	100.10 ± 1.17
Analyst-II	98.76 ± 0.83	100.22 ± 0.86
Accuracy [% Recovery]	99.57-100.82	97.87 - 99.20
Precision		
Repeatability [n=6]	0.58	0.17
Intra-day [n=3]	0.43 - 0.69	0.56 - 0.73
Inter-day [n=3]	0.98-1.17	0.42 - 0.89
Sensitivity(µg)		
Limit of detection	49.87	0.38
Limit of quantification	166.23	1.26
Robustness	Robust	Robust

Table 4 Summary of the assay parameters for HPTLC and UV-AUC studies using regression, validation, and in-house injection formulation.

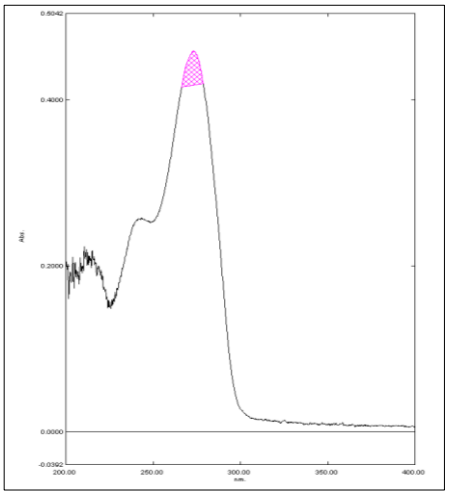

Figure 1 Zero order spectrum of cidofovir standard depicting AUC in between 266.4 and 278.8 nm

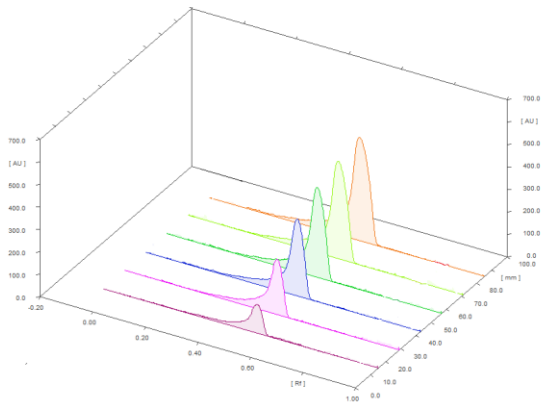

Figure 2 Linearity studies for Cidofovir dichloromethane: methanol: triethylamine (3:2:0.5 *v/v/v*) as mobile phase during HPTLC.

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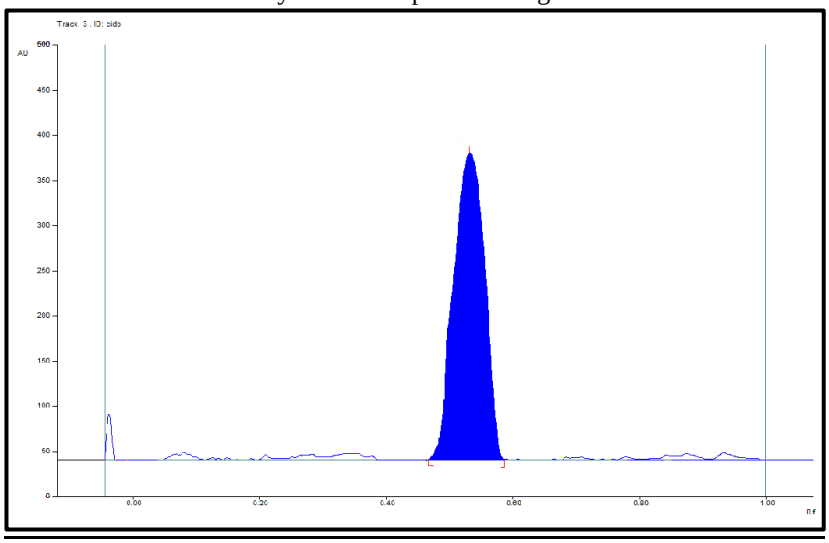

Figure 3 HPTLC chromatogram for cidofovir standard in dichloromethane: methanol: triethylamine (3:2:0.5 *v/v/v*) as mobile phase.

4. CONCLUSION

To accurately estimating CIDO in bulk and custom compositions, a new HPTLC method was devised. The ICH recommendations were used to establish the method's validity, and it was shown to have the following qualities: accuracy, linearity, robustness, simplicity, and affordability.

5. ACKNOWLEDGMENTS

The facilities available to execute this research study are gratefully acknowledged by the authors to Dr. S.J. Surana, Principal, R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur (MS), India.

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