ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Use of Eosin for Spectroscopic Determination of Adefovir Dipivoxil

Shaimaa Elsayed [a,b*], Alaa El-Gindy [b], Yasser El-Shabrawy [a,c], ElSayed A. Ibrahim [b]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Horus University-Egypt, New Damietta, Egypt; [b] Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt; [c] Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

Received: 08. 03. 2024

Revised: 23. 03. 2024

Accepted: 28. 03. 2024

*Correspondence Author:

E-mail address:

shelsied@horus.edu.eg

Abstract

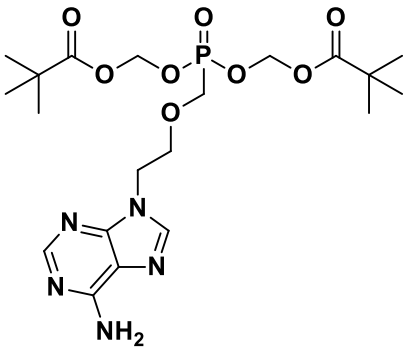
This paper elucidates a new spectrofluorometric method for the determination of Adefovir Dipivoxil in its pharmaceutical dosage form using Eosin, the colored ion - pair complex was produced at λ em 544 nm and excitation at λ ex 267 in acetate buffer solution with a pH of 3.5. The linearity range was 0.1 – 1.5 µg/ml, with a percentage recovery of 99.31, and detection limit of 0.032 µg/ml. The quantification limit was 0.097 µg/ml. The method was successfully applied to determine Adefovir Dipivoxil in its pharmaceutical dosage form. The method was Sensitive, rapid and cost-effective, demonstrating ease of application suitable for routine quality control analysis.

Keywords: Eosin, Spectroscopic Determination, Adefovir Dipivoxil.

1. Introduction

Adefovir Dipivoxil (ADV) (Fig. 1) or (bis(POM)-PMEA), is known chemically as

9-[2-({bis[(pivalaloyloxy)methoxy]phosphinyl]} methoxy) ethyl]adenine (Darsazan et al., 2017). ADV is a diester prodrug of the active moiety Adefovir, which is an acyclic nucleotide analogue of adenosine monophosphate (Alqahtani et al., 2023). ADV is a reverse transcriptase inhibitor used for the treatment of chronic hepatitis B. Following absorption, ADV is converted into Adefovir and then phosphorylated to Adefovir diphosphate in hepatocytes, which is a competitive inhibitor of hepatitis B virus (HBV) polymerase (Lee and Martin, 2006). Long-term ADV use helps achieve and maintain viral suppression, regression of fibrosis, and reversal of cirrhosis in most patients (Wang et al., 2014).



Adefovir Dipivoxil

Fig 1. Structure of ADV

The oral bioavailability in fasted patients is approximately 25%. Administration of food (high fat meal containing 40 to 50% fat) increases the oral bioavailability, with an increase in the AUC of approximately 40% (Wassner et al., 2020).

Eosin–Y [2-(2, 4, 5, 7-tetrabromo-6-oxido-3-oxo-3H-xanthen-9-yl) benzoate] Fig.2. as a halo fluorescein dye has been used as an ion-pairing agent for the spectrophotometric determination of some important pharmaceutical compounds with or without extraction (Hamad et al., 2022).

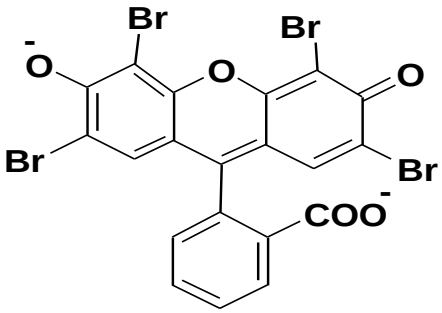


Fig 2. Eosin-Y M.wt.691.86

2.Experimental:

2.1. Instruments:

- Jasco FP6200 single beam spectrofluorometer (Japan).
- Jenway 3150 pH meter, (Jenway, USA).
- Analytical balance (Precisa125A, Switzerland).

2.2. Materials and Reagents:

Pure ADV with purity of 99.96% was supplied by EVA Pharma. FODAVIR 10 mg tablet was obtained from a local hypermarket. Batch number of FODAVIR is 2202098A containing 10 mg ADV per tablet, EVA Pharma, Cairo, Egypt. All reagents were of analytical grade and solvents were of spectroscopic grade, distilled water was used throughout the procedure.

Eosin-Y (Sigma; Aldrich, Germany). (2 x 10^{-5} M and 4.39 x 10^{-6} M) aqueous solution, methanol, acetic acid were purchased from (El-Nasr Company, Abu- Zaabal, Egypt).

Preparation of an acid phthalate buffer from pH (2.5 - 3.5): place 50 ml of 0.2 M potassium hydrogen phthalate in a 200 ml volumetric flask, adjust to the required pH with 0.2 M hydrochloric acid or 0.2 M sodium hydroxide using pH meter and then add water to volume with distilled water. Acetate buffer 0.2 M, pH (٢.5 - 4.5) was prepared by mixing suitable volumes of 0.2M sodium acetate and 0.2 M acetic acid and adjusting the pH using pH meter.

2.3. Standard Solutions

Ten mg of ADV powder was accurately weighed and transferred into 100 ml volumetric flask, then dissolved with the least amount of methanol, the volume was completed to the mark with water to obtain (0.1 mg ml^{-1}) drug concentration from which the working standard (0.01 mg ml^{-1}) was prepared by dilution with water and solution was stable in refrigerator for one month.

2.4. Procedures:

2.4.1. Spectral characteristics:

Upon reacting a definite concentration of ADV with Eosin-Y in the presence of acetate buffer (pH 3.5), a highly colored ion - pair complex was formed. It was found that the formation of this complex reduces the fluorescence intensity of Eosin-Y.

The quenching in the fluorescence intensity of blank was monitored at λ em 544 nm after excitation at λ ex 267 nm, by measuring the blank first then the experiment and the difference in the fluorescence intensity between them is calculated as shown in **figure (3)**.

2.4.2. Optimization of the reaction conditions:

I- Effect of pH:

Accurately measured aliquot of the drug equivalent to (0.012 mg) was transferred into a series of 10 ml volumetric flasks and diluted to about 6 ml with water then 1 ml of (2 $X10^{-5}$M) Eosin-Y solution was added and the mixture was mixed well before the addition of 1.5 ml of acetate buffer with different pH ranging from (2.5 - 4.5). The flasks were completed to volume with water and then the difference in the relative fluorescence intensities between blank and test solutions was measured at λ em 544 nm after excitation at λ ex 267 nm.

For the highest difference in the relative fluorescence intensities and sensitivity, the buffer solution should be added after mixing the drug-dye solution at neutral pH. It was found that, maximum difference in the relative fluorescence intensities was obtained upon using acetate buffer pH 3.5, as shown in **figure (4).**

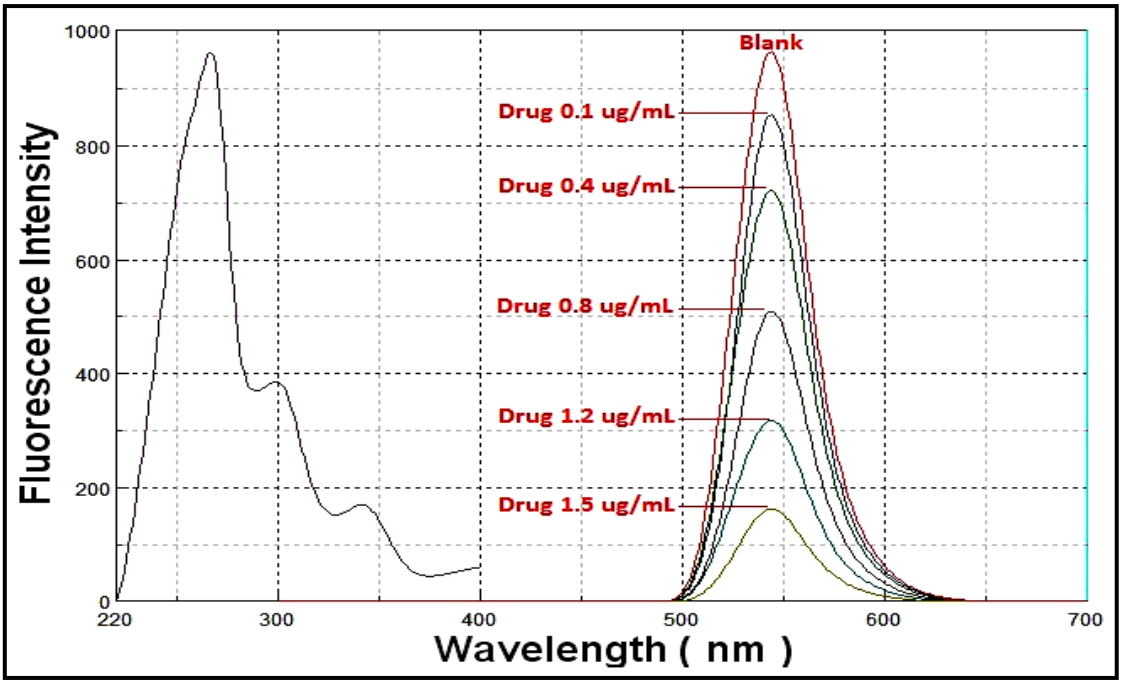


Figure (3): Excitation and Emission Spectra of the Reaction Product of ADV (0.1 - 1.5 µg ml^{-1}) with Eosin- Y.

II- Effect of buffer volume:

The procedure under "**i-Effect of pH**" was repeated using different volumes (0.5- 4 ml) of acetate buffer pH 3.5. It was revealed that 1.5 ml of the buffer solution was sufficient to give a maximum difference in the relative fluorescence intensities, as shown in **figure (5)**.

III- Effect of Eosin-Y volume:

The procedure detailed under **"i-Effect of pH"** was followed using 1.5 ml of acetate buffer pH 3.5 and different volumes of Eosin-Y (2 x 10^{-5}M) ranging from (0.25 – 2ml). It was revealed that 1ml of the dye was sufficient to give maximum difference in the relative fluorescence intensities, as shown in **figure (6).**

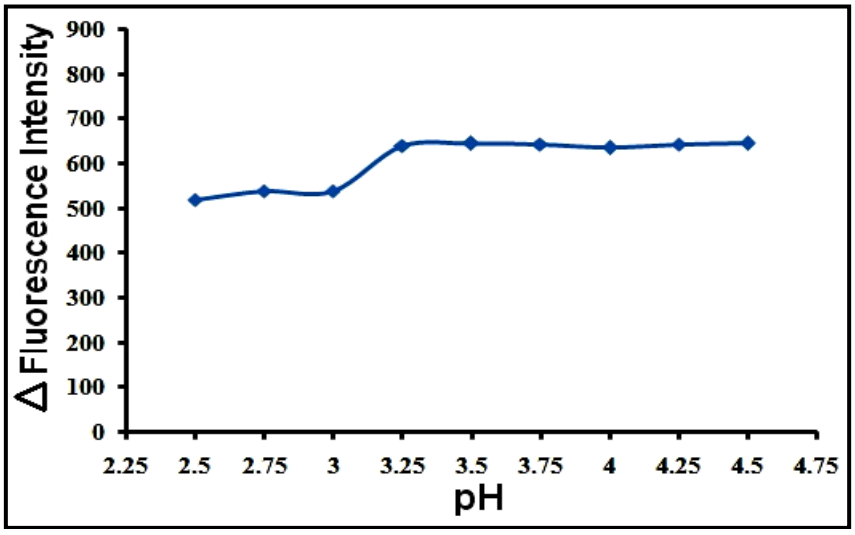


Figure (4): Effect of pH on The Fluorescence of ADV (1.2 µg ml^{-1}) Reaction Product with Eosin - Y at λem 544 nm.

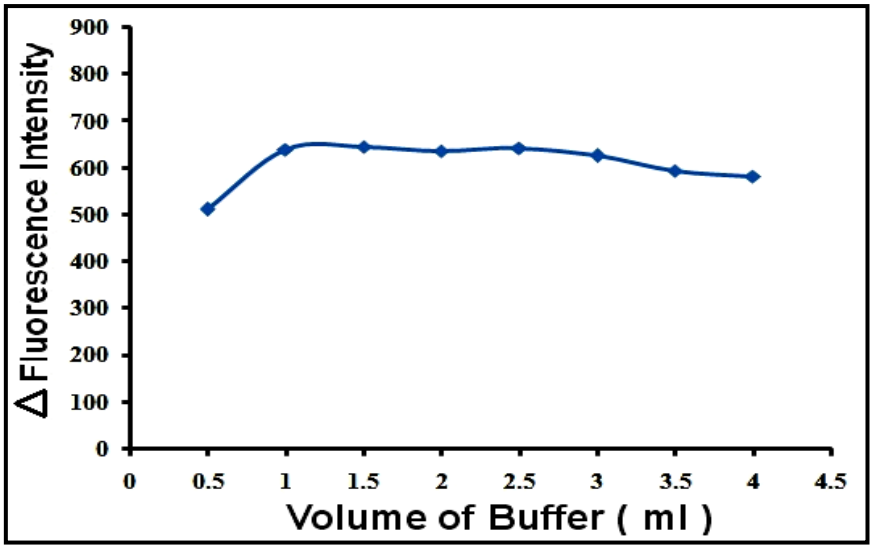


Figure (5): Effect of Buffer Volume on the fluorescence of ADV (1.2 µg ml^{-1}) Reaction Product with Eosin - Y at λem 544 nm.

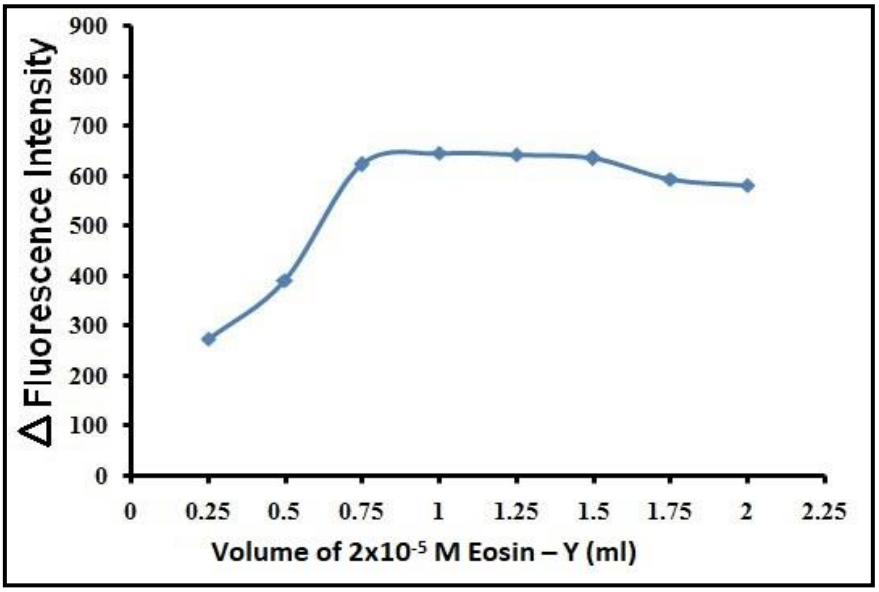


Figure (6): Effect of Eosin - Y Volume on The Fluorescence of ADV (1.2 µg ml^{-1}) Reaction Product with Eosin - Y at λem 544 nm.

2.4 .3.Determination of the Stoichiometry of the Reaction:

Continuous variation (job's method)(MOHAMED et al., 2020)

In a series of 10 ml volumetric flasks, (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.5 ml) of (4.39 x 10^{-6} M) ADV were transferred, then (4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5 ml) of Eosin - Y solution (4.39 x 10^{-6} M) were added (the sum of ADV and reagent equals to 5 ml). The volume in each flask was adjusted with distilled water to 8 ml then 1.5 ml of acetate buffer pH 3.5 was added then volumes were completed to the mark with water. The difference in fluorescence intensity of the formed ion pair complexes with Eosin -Y were measured at λ em 544 nm after excitation at λ ex 267 nm against the appropriate reagent blank, as shown in **figure (7).**

2.4.4. Method Validation:

2.4.4.1. Linearity:

Aliquots of drug solution of (0.01 mg ml^{-1}) containing (1 – 15 µg) were transferred into a series of 10 ml volumetric flasks; the volumes were diluted to 6 ml with water. Eosin-Y 1 ml of (2 x 10^{-5} M) solution was then added and the mixture was mixed well before addition of 1.5 ml of acetate buffer pH 3.5. The mixture was adjusted to the volume with water then the difference in fluorescence intensity between the reagent blank and each experiment was measured at λ_{em} 544 nm after excitation at λ ex 267 nm, as shown in **figure (8) and table (1) .**

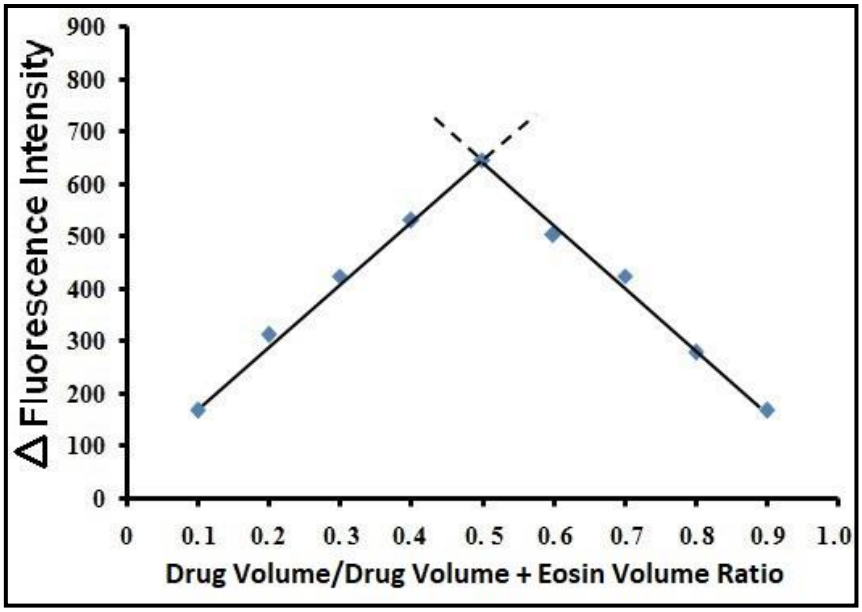


Figure (7): Stoichiometry of The Reaction of of ADV (4.39 x 10^{-6} M) With Eosin –Y (4.39 x 10^{-6} M) by Continuous Variation (Job's) Method at λ em 544 nm.

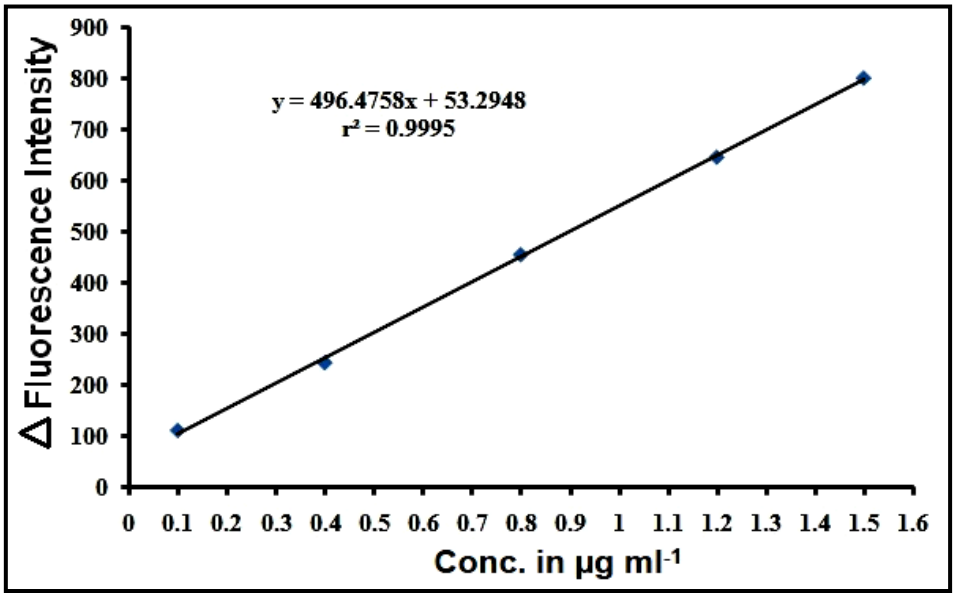


Figure (8): Calibration Graph of ADV with Eosin – Y at λ_{em} 544 nm.

Table (1): Selected Spectral data for the Determination of ADV by The Proposed Eosin-Y Procedure:

Parameters	Proposed method
λ_{ex} and λ_{em}	267 and 544 nm
Linearity range (µg ml^{-1})	0.1 – 1.5
LOD (µg ml^{-1})	0.032
LOQ (µg ml^{-1})	0.097
- Regression Equation **- Slope ± S.D** **- Intercept ± S.D**	**ΔF^{*}** = 496.4758 C^{**} + 53.2948 496.4758 ± 4.521 53.2948 ± 7.158
Correlation coefficient (r)	0.9995

ΔF^{*} is fluorescence intensity difference, C^{**} is concentration in µg ml^{-1}.

2.4.4.2. Accuracy and Precision:

I- Intra-Day (Within Day) Precision:

Three replicate determinations of different concentrations (0.4, 0.8 and 1.2 µg ml^{-1}) of the drug within the linearity range were done. The reaction procedure was followed as in linearity 2.4.4.1**(Table 2)**.

II- Inter-Day (Between-Day) Precision:

The procedure described under "i- Intra-Day" was repeated for three days. Accuracy was expressed as recovery percent (R%) (100%, 99.34%, 101.42%) and precision was expressed as percent relative standard deviation (RSD%) (0.762%, 0.153%, 0.371%).The results are listed in **Table 2**.

Table (2): Intra-Day and Inter-Day Accuracy and Precision for the Determination of ADV by the Proposed Procedure:

Conc. µgml^{-1}	Intra-Day			Inter-Day		
	Found Conc. ± SD	**Accuracy (Recovery%)**	(Precision) **(RSD%)**	**Found Conc. ± SD**	**Accuracy (Recovery%)**	**Precision (RSD%)**
0.4	0.40±0.003	100.00	0.842	0.40±0.003	100.00	0.762
0.8	0.80±0.003	100.00	0.326	0.79±0.001	99.34	0.153
1.2	1.22±0.003	101.65	0.265	1.22±0.005	101.42	0.371

2.4.4.3. Specificity:
Specificity was determined by application of standard addition technique to check the effect of the tablet matrix on the determination of the drug. The obtained results, **Table (3)**, proved that the proposed method could selectively analyze the drug without any interference from any tablet excipients.

2.4.4.4. Stability:
I. Stability of Test Solution:
Two solutions of ADV (0.1 mg ml^{-1}) were prepared and one of them was kept at room temperature while the other was kept in refrigerator. The solutions were determined every hour for first 12 hours and then every day using freshly prepared solution of (2 x 10^{-5} M) Eosin – Y. The results were compared with those of freshly prepared test solution of the same concentration. The solution of drug was stable at room temperature for 2 days and stable at fridge for at least one month.
II. Stability of Reagent Solution:
Two solutions of (2 x 10^{-5} M) Eosin – Y were prepared one of them was kept at room temperature while the other was kept in refrigerator. The solutions were used for the determination of the same concentration of freshly prepared solution ADV every one hour for first 12 hours and then every day and the results were compared with those obtained with freshly prepared reagent. The Eosin solution was stable at room temperature for 5 days and stable at fridge for at least one month.

2.4.4.5. Determination of ADV in Pharmaceutical Preparation Using the Proposed Eosin - Y Method:
Ten **Fodavir** 10 mg tablets were accurately weighed and finely powdered, then a quantity equivalent to 10 mg of **Fodavir** was shaken three times with 25 ml methanol for 15 minutes then filtered into 100 ml volumetric flask and the volume was completed to the mark with water to obtain a concentration of (0.1 mg ml^{-1}). The solution was analyzed as the procedure of linearity **(2.4.4.1).**

2.4.4.6. Accuracy:
Aliquots of standard *ADV* solution (0.01 mg ml^{-1}) containing (4 –10 **µg**) were added to a series of 10 ml volumetric flask containing (4 **µg**) of already analyzed **Fodavir** 10 mg tablets. One ml of (2 X 10^{-5} M) Eosin-Y solution and 1.5 ml acetate buffer pH 3.5 were added. The volume was completed to 10 ml with water. The procedure of measurement was follow as linearity **(2.4.4.1)**. Concentration of added ADV were determined. Results are presented in **Table 3.**

Table (3): Recovery Study of ADV by Adapting Standard Addition Technique via Reaction with Eosin-Y in its Tablets:

Fodavir® 10 mgTablets			
Taken µg ml^{-1}	**Pure added µg ml^{-1}**	**Pure found µg ml^{-1}**	**Recovery %**
0.4	0.4	0.40	100.00
	0.8	0.79	99.31
	1	0.99	99.89
Mean			99.73
RSD%			0.372

3. Results & Discussions

The present study depends on that**,** Eosin - Y dye is an anionic halo fluorescein derivative that strongly associated with ADV in acidic medium to form a highly stable ion – pair associate. The supposed reaction pathway was shown in the following scheme **Fig 9.**

This complex was probably formed via the electrostatic interaction between the amino group of the drug and the carboxylate anion of the dye. This might primarily occur in acidic solutions (Rahman, 2017).

ADV reacts with Eosin – Y dye in the presence of acetate buffer (pH 3.5) to form a highly stable ion – pair associate that leads to quenching in the fluorescence intensity of Eosin - Y at emission wavelength 544 nm after excitation at 267 nm as shown in **Fig 3.**

Different parameters involved in the reaction were studied such as buffer pH, buffer volume, concentration of the reagent. The effect of pH using acid phthalate buffer from pH (2.5 - 3.5) and acetate buffer from pH (2.5 - 4.5) as well as the buffer volume were studied and revealed that 1.5 ml of pH 3.5 acetate buffer were sufficient to give maximum quenching effect **figure (4,5),** while the acid phthalate buffer give unacceptable result. The effect of Eosin – Y volume was also studied, **Fig.6** revealed that, 1 ml of (2 X 10^{-5}M) were sufficient.

Continuous variation (Job's method) method was applied for the determination of stoichiometry of ADV using concentration of (4.39 x 10^{-6} M) for the drug and Eosin – Y. The drug: reagent ratio was found to be 1:1, as shown in **Fig.7.**

Calibration graph was constructed for the determination of ADV by the proposed procedure where Beer's law was obeyed in the range of 0.1 – 1.5 µg ml^{-1} with 2 X 10^{-5} M Eosin - Y dye, **figure (8).** The linear regression of the graph was as follows.

ΔF_{544nm} = 496.4758 C + 53.2948 (r=0.9995)

Where **ΔF** is the difference in fluorescence intensity at 544nm, **C** is the drug concentration in µg ml^{-1}.

The ICH Q2 (R1) instructions (Borman and Elder, 2017) for determination of the limit of detection (LOD) and limit of quantification (LOQ) were applied using the following equations:

LOQ = 10 Sa/b

LOD = 3.3 Sa/b

Sa is the standard deviation of the intercept of the calibration curve and b is the slope of the calibration curve.

Table (1), illustrated the regression parameters of the calibration curve and correlation coefficient, working range, **LOD**, **LOQ** of the drug obtained with the drug analyzed.

Within - day accuracy and precision was tested by the determination of ADV at three levels (0.4, 0.8 and 1.2 µg ml^{-1}). The accuracy was represented as percentage recovery **(R %)** and it was ranged from 100.00 to 101.65. While, precision was represented as percentage relative standard deviation **(RSD %)** and it was ranged from 0.265 to 0.842.

Between - day accuracy and precision was performed by adopting the within - day procedure for different three days. The between - day accuracy was ranged from 100.00 to 101.42**%**. While, precision was ranged from 0.371to 0.762, as shown in **table (2)**.

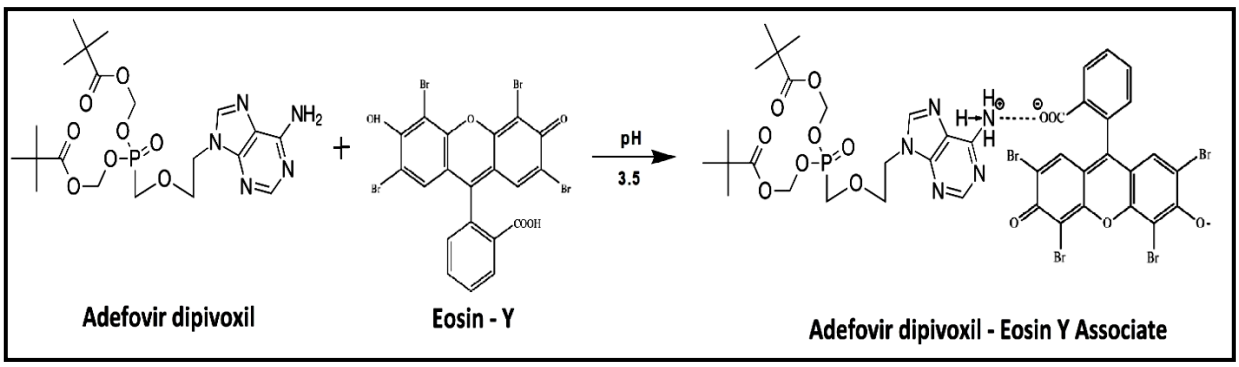


Fig 9. The supposed reaction pathway between ADV and Eosin - Y

Table (4): Statistical Analysis of the Results Obtained by the Proposed and Reported Procedures for the Determination of ADV in Its Pharmaceutical Preparation:

Parameters	Fodavir ® 10 mg tablets	
	Proposed Method	**Reported method* (Bhirud and Hiremath, 2022)**
Number of measurements	5	5
Mean % found	99.31	100.48
SD (standard deviation)	0.916	1.314
Variance	0.827	1.743
Student's *t*-test	1.636 (2.306) **	——
***F*-value**	2.107 (6.388) **	——

* UV Spectrophotometric method using methanol as solvent and λ_{max} of the drug was found to be 260 nm; ** The values in parenthesis are tabulated values of "*t* " and "*F* " at (P = 0.05)

The proposed method was used for determination of ADV in **Fodavir** ® 10 mg tablets with mean percentage recovery ± RSD% 99.31 ± 0.9 16. as shown in **table (4).**

Statistical comparison between results obtained by applying the proposed procedure and those obtained by applying the reported method(Bhirud and Hiremath, 2022) For determination of ADV in **Fodavir** ® 10 mg tablets. Good results were obtained with less calculated t and F values than the tabulated ones revealing no significant difference in accuracy and precision, **table (4).**

Recovery study was performed by adopting standard addition technique. Different concentrations of standard ADV (0.4 –1**µg/ml**) were added to previously analyzed **Fodavir** ® 10 mg tablets (0.4 **µg/ml)**. Results are presented in **table (3)** and revealed that, the proposed method with high recovery.

4. Conclusion:

Sensitive, rapid and cost-effective spectroscopic method based on the reaction of ADV with Eosin was carried out and optimized. The method showed good validation criteria according to official guidelines. Additionally, comparison with previously published spectroscopic method revealed no significant difference between them. The method proved to be Sensitive, rapid and cost-effective, demonstrating ease of application suitable for routine quality control analysis.

4. Conclusion:

Sensitive, rapid and cost-effective spectroscopic method based on the reaction of ADV with Eosin was carried out and optimized. The method showed good validation criteria according to official guidelines. Additionally, comparison with previously published spectroscopic method revealed no significant difference between them. The method proved to be Sensitive, rapid and cost-effective, demonstrating ease of application suitable for routine quality control analysis.

5. References:

ALQAHTANI, T., KUMARASAMY, V., ALGHAMDI, S. S., SULIMAN, R. S., BIN SALEH, K., ALRASHED, M. A., ALDHAEEFI, M. & SUN, D. 2023. Adefovir Dipivoxil as a Therapeutic Candidate for Medullary Thyroid Carcinoma: Targeting RET and STAT3 Proto-Oncogenes. *Cancers*, 15**,** 2163.

BHIRUD, C. H. & HIREMATH, S. N. 2022. Method Development and Validation for the Estimation of Adefovir Dipivoxil in Bulk and Pharmaceutical Dosage Forms and Its Stress Degradation Studies Using UV-Vis Spectrophotometric Method: Experimental Investigation. *Current Aspects in Pharmaceutical Research and Development Vol. 9***,** 89-99.

BORMAN, P. & ELDER, D. 2017. Q2 (R1) validation of analytical procedures: text and methodology. *ICH quality guidelines: an implementation guide***,** 127-166.

DARSAZAN, B., SHAFAATI, A., MORTAZAVI, S. A. & ZARGHI, A. 2017. A simple and specific stability-indicating RP-HPLC method for routine assay of adefovir dipivoxil in bulk and tablet dosage form. *Iranian journal of pharmaceutical research: IJPR*, 16**,** 132.

HAMAD, A. A., ALI, R. & DERAYEA, S. M. 2022. A simple single jar "on–off fluorescence" designed system for the determination of mitoxantrone using an Eosin Y dye in raw powder, vial, and human biofluids. *RSC advances*, 12**,** 7413-7421.

LEE, W. A. & MARTIN, J. C. 2006. Perspectives on the development of acyclic nucleotide analogs as antiviral drugs. *Antiviral research*, 71**,** 254-259.

MOHAMED, S. H., ISSA, Y. M. & SALIM, A. I. 2020. QUANTITATIVE DETERMINATION OF CLOPIDOGREL BISULFATE USING VALIDATED SPECTROPHOTOMETRIC METHODS. *Asian Journal of Advances in Research***,** 180-190.

RAHMAN, H. 2017. Utilization of Eosin dye as an ion pairing agent for determination of pharmaceuticals: A brief review. *Int. J. Pharm. Pharm. Sci*, 9.

WANG, X., ZHANG, C., ZHU, Y., XIONG, Y. & WANG, Y. 2014. Efficacy of 2 years of entecavir plus adefovir therapy in patients with chronic hepatitis B who had failed on prior nucleos (t) ide analog treatment. *Antiviral research*, 103**,** 71-77.

WASSNER, C., BRADLEY, N. & LEE, Y. 2020. A review and clinical understanding of tenofovir: tenofovir disoproxil fumarate versus tenofovir alafenamide. *Journal of the International Association of Providers of AIDS Care (JIAPAC)*, 19**,** 2325958220919231.