EXTRACTIVE SPECTROPHOTOMETRIC METHODS FOR THE ASSAY OF OSELTAMIVIR PHOSPHATE, FAMCICLOVIR AND ACYCLOVIR IN PURE AND DOSAGE FORMS

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ABSTRACT

For the determination of oseltamivir phosphate (OSP), acyclovir (ACL), and famciclovir (FMV) medicines in pure form and their formulations, quick, accurate, and simple spectrophotometric procedures have been established. The procedures were relied on the formation of ion-pair complexes between the drugs and anionic dyes (OSP with bromothymol blue (BTB), ACL with methyl red (MTR) and FMV with methyl orange (MTO)) in an acidic medium, separately. The yellow OSP-bromothymol blue complex, orange ACL-methyl red complex and orange-yellow FMV-methyl orange complex formed were quantitatively extracted with chloroform and their absorbance measured at 416, 416 and 488 nm wavelengths, respectively. The limits of detection and quantification were found to be 0.5, 1.5, 1.0 µg mL^{-1} and 1.7, 5.0 and 3.3 µg mL^{-1} for the OSP, ACL and FMV, respectively. The linearity was established from 1-5, 2-12 and 5-14 µg mL^{-1} for OSP, ACL and FMV, respectively. R^2 (regression coefficient) values were above 0.996 for all the three drugs. Recovery (accuracy) results were found within 98.0 % to 102 %. The developed methods comply with the ICH guidelines. The developed methods were successfully used to measure OSP, ACL and FMV in both their pure and in formulation forms.

Keywords: Oseltamivir phosphate, acyclovir, famciclovir, extraction, spectrophotometric determination

INTRODUCTION

The chemical name of oseltamivir phosphate (OSP) is [(3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester, phosphate] (Fig.1), which is commonly employed in prevention and treatment of influenza virus A and influenza virus B. The neuraminidase inhibitor oseltamivir carboxylate is an ethyl ester prodrug, which is an active metabolite in the liver of a human[1]. Influenza is an extremely transmittable viral disease, generally severe in winter season, and it is responsible for acute respiratory disease due to epidemic and pandemics virus which causes significant morbidity and mortality all over the world[2]. Acyclovir (ACL), [2-amino-1,9-dihydro-9-[(2-hydroxyethoxy) methyl]-6*H*-purina-6-one; acycloguanosine; 9-[2-hydroxyethoxy) methyl]guanine, (Fig.1) , is frequently used to treat Herpes simplex (HSV) types I and II as well as varicella viruses and this is extremely energetic in *in vitro* conditions, but less toxic in mammalian cells. ACL gets phosphorylated to became the active compound ACL triphosphate. Intracellular conversion of ACL to triphosphate by viral thymidine kinase takes place when ACL is entering into a herpes infected cell, which initiates inhibition of the HSV at a specific DNA polymerase, followed by viral DNA-synthesis without disturbing common cellular processes[3,4]. ACL is highly discriminating and low in cytotoxicity[5]. Famciclovir (FMV) (Fig. 1) is an analogue of an acyclic guanine nucleoside and is chemically 2-[2-(2-amino-9*H*-purin-9-yl) ethyl] trimethylene diacetate. It is effective against HSV types 1 and 2, especially varicella-zoster virus (VZV)[6-10].

A broad wide literature survey revealed that there are a few analytical methods for the determination of OSP, ACL and FMV. Isocratic RP-HPLC method was developed to determine the OSP in capsules as well as in blood plasma[1]. The reaction of the drug with 4-chloro-7-nitrobenzofurazan in a borate buffer solution with a pH of 8.50 served as the basis for the development of this method. Stationary phase used was a C_{18} column and

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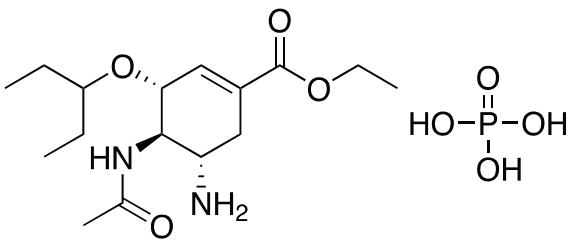
mobile phase was acetonitrile and 10 M mol nitric acid of pH 3.0. The results were obtained with fluorescence detection at 470 nm and 541 nm. The estimation of ACL in its formulations by a spectrophotometric method has been developed[3]. In this procedure, 3-methylbenzothiazolin-2-one hydrazone (MBTH) was used for the oxidative coupling reaction to produce deep-green colored species absorbed at 616 nm. UV spectrophotometric determination of ACL in tablet formulation methods has been developed and validated[4]. The maximum absorption in ultraviolet range was at 252 nm. Linearity range was established at 1-30 µg mL^{-1} and recovery parameters produced at the concentration of 80, 100 and 120%. The obtained recovery outcomes were found to be in the range of 98.458 - 101.984%. Determination of FMV in bulk and tablet dosage form by UV spectrophotometric method has been

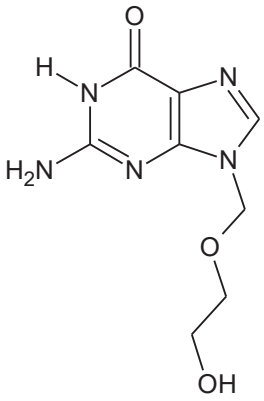
Table I: Comparison of the spectrophotometric methods for the determination of OSL, ACL AND FMV

Sl. No.	Drug(s) analysed	Reagent	λ_{max}, nm	LOD	Accuracy	R^2	Remark	Ref No.
1	ACL	3-Methyl benzothiazolin-2-one hydrazone and $FeCl_3$ as oxidant	616 nm	NA	99.75%	0.9998	Not included OSP and FMV	3
2	ACL	Derivatization method	252, 238 259 and 288 nm	NA	98.458 to 101.984%	0.9971, 0.9981, 0.9969 and 0.9951	Not included OSP and FMV	4
3	FMV	2,3-dichloro-5,6-dicyano-p-benzoquinone	415 nm	NA	100.24 and 100.38%	0.9993	Not included OSP and ACL	6
4	FMV	Orange II and alizarin red S	480 and 440 nm	0.63 and 0.66 µg mL^{-1}	100.3, 100.8, 100.8 and 99.8%	0.9994 and 0.9993	Not included OSP and ACL	7
5	OSP	Congo red and bromochlorophenol blue	520 and 590 nm	NA	NA	0.998 and 0.957	Not included ACL and FMV	12
6	FMV	Bromocresol green, bromothymol blue, bromocresol purple and bromophenol blue	418, 412, 407 and 414 nm	0.15, 0.16, 0.23 and 0.14 g mL^{-1}	99.84 to 100.26%	0.9997	Not included OSP and ACL	13
7	ACL	Ninhydrin-ascorbic acid	540 nm	0.3 µg mL^{-1}	96.9 to 102.0%	0.9930	Not included OSP and FMV	14
8	ACL	Cerium(IV) ammonium sulphate	363 nm	78.15	99.10	0.9997	Not included OSP and FMV	15
9	OSP, ACL and FMV	BTB, MTR and MTO	416, 488 and 416 nm	0.5, 1.5 and 1.0 µg mL^{-1}	101.35, 100.50 and 99.0%	0.996, 0.997 and 0.999	Included OSP, ACL and FMV	Proposed method

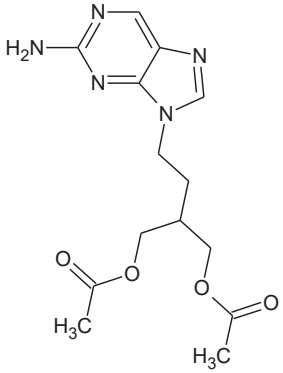
NA-Not Available



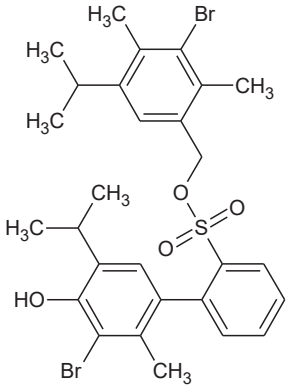
Oseltamivir phosphate (OSP)



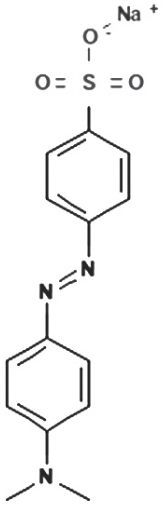
Acyclovir (ACL)



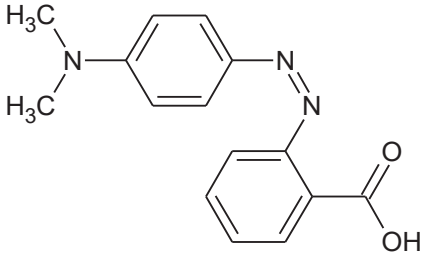
Famciclovir (FMV)



Bromothymol blue (BTB)



Methyl orange (MTO)



Methyl red (MTR)

Fig. 1: Chemical structures of OSP, ACL, FMV, BTB, MTO and MTR

developed[6]. The procedure was based on the reaction of 2, 3-dichloro-5,6-dicyano-p-benzoquinone as the acceptor and FMV as the n-electron donor. The resultant intense colored solution absorbed at 415 nm. The determination of FMV in bulk and its dosage form was demonstrated by two methods, A and B, which relied on the formation of ion-pair complexes of the drug and dyes, such as orange II and alizarin red taking place in acidic medium[7]. Further extraction was performed with chloroform to establish colored chromogen which was absorbed in visible region at 480 nm and 440 nm, respectively. Determination of FMV based on the derivatization reaction of FMV with fluorescamine by spectrofluorimetric method was developed in pure and pharmaceutical preparation[10].

From the literature survey, it was noticed that no extractive spectrophotometric method was reported for OSP and ACL. The reported spectrophotometric method for the determination of FMV is laborious. Table I summarizes the comparative studies of the reported and proposed methods. It was noticed that no spectrophotometric method was found for the determination of all the three drugs *viz.,* OSP, ACL and FMV. In view of this, we developed spectrophotometric techniques for the estimation of OSP, ACL and FMV. The production of ion-complexes by interactions between the drugs and acidic dyes served as the foundation for the proposed extraction methods. Such complex can be quantified and measured at particular visible region wavelengths. The proposed methods are economic, simple, accurate and precise.

MATERIALS AND METHODS

Instrument

The method was developed with the UV-Visible spectrophotometer, Agilent, Cary 60 model, consisting of single beam with deuterium lamp used as the source of light. The spectra were processed and monitored by WinLab software. Eutech pH meter was used for the pH adjustment for the acidic medium (KCl:HCl buffer pH-2.2). The experimental solvents were degassed in all ultra-sonic bath sonicator.

Reagents and chemicals

All substances-drugs and chemicals-used were more than 98.0% pure. The reference standards for OSP, ACL, and FMV were provided as gift samples by KAPL India Ltd., Bengaluru. The dosage forms, Tamiflu (Roche Products Ltd), Tascort 400 (Psychocare Health Pvt. Ltd.) and Virovir 500 (FDC Ltd.) for OSP, ACL and FMV, respectively, were purchased from local pharmacy to carry out the assay parameter. BTB, MTR and MTO were procured from Loba Chemie Ltd., Mumbai. The analytical grade chloroform and hydrochloric acid were procured from the Merck Ltd., Mumbai, India. Potassium chloride, sodium hydroxide and anhydrous sodium sulphate (drying agent) were purchased from SD Fine Chemicals, India. Ultra-purified water was obtained from Siemens, India.

Table II: Linearity and outcomes, limit of detection, limit of quantification and system suitability

Parameter	OSP	ACL	FMV
Linear dynamic range	1.0-5.0	2.0-12	5.0-14.0
Slope	0.046	0.45	0.073
Intercept	0.146	0.653	0.050
Correlation coefficient (R^2)	0.997	0.999	0.996
LOD (µg mL^{-1})	0.5	1.5	1.0
LOQ (µg mL^{-1})	1.7	5.0	3.3
% RSD*	0.44	0.62	0.75
Wavelength	416	416	488

** Average of 9 determinations*

Preparation of standard solution

The preparation of standard solution was as follows. Accurately weighed reference standards of OSP, ACL and FMV were transferred into three 100 mL standard flasks, separately and filled with methanol up to the specified limit for OSP and FMV and with dimethyl formamide for ACL. Then 0.1 mL of each of this solution was taken into new, 100 mL standard flasks and made up to the mark with respective solvents. The resultant solution concentration was 1.0 µg mL^{-1}. To create the calibration curve, the stock solution was diluted to the necessary concentration.

METHOD DEVELOPMENT

In a 250 mL separating flask the desired dilutions of reference standard solutions were pipetted out separately, the followed by addition of standardized 20 mL acidic buffer KCl:HCl, pH-2.2 buffer, 1.0 mL of 0.1% BTB, 0.5 mL of 0.1% MTR and 2.0 mL of 0.1% MTO for the OSP, ACL and FMV, respectively and then extracted with 10 mL of organic solvent, chloroform. The mixture of solution was shaken vigorously for 2 mins for four times; the layers for were allowed to separate. After the separation, the lower layers of organic solvent were yellow, yellowish orange and yellow colored ion-pair complex solutions for OSP, ACL and FMV, respectively. These solvents

Table III: Accuracy and precision results

Concentration	Amount of drug taken	OSP	% RSD*	ACL	% RSD*	FMV	% RSD*	Limit
60%	60 mg mL^{-1}	101.0	0.45	100.5	0.62	101.5	0.54	98-102%
80%	80 mg mL^{-1}	99.0	0.62	99.5	0.73	99.7	0.71	98-102%
120%	120 mg mL^{-1}	99.2	0.91	99.7	0.84	99.3	0.82	98-102%
Intra day	-		0.67	-	0.82	-	0.65	NMT-2.0
Inter day	-		0.49	-	0.78	-	0.57	NMT-2.0

** Average of 9 determinations*

Table IV: Assay results of OSP, ACL and FMV with marketed formulation

Drug	Label claim	Obtained result	Assay value*	Limits
OSP	75 mg	74 mg	101.35%	98-102%
ACL	400 mg	398 mg	100.50%	98-102%
FMV	500 mg	495 mg	99.00%	98-102%

** Average of 9 determinations*

were collected separately and passed through the drying agent, anhydrous sodium sulphate. The aqueous free solution was taken for the measurement in the UV- visible spectrophotometer. Meanwhile, the corresponding blanks were measured with respective dye and buffer except reference drug. There was no absorbance found in the blank solution in all the three cases. Hence, respective drugs were developed the ion pair complex with BTB, MTR and MTO (Fig. 1) for the OSP, ACL and FMV, respectively.

Optimization of extraction conditions

Selection of organic solvent for extraction

For the proposed method, the selection of right organic solvent is an important step in the extraction process. The organic solvents *viz.,* diethyl ether, chloroform, dichloromethane and carbon tetra chloride were examined. With chloroform as organic solvent, a clear separation between the aqueous and organic solvent was found and color intensity of the respective ion-pair complexes were good. No good separations were observed with other solvents, hence, extraction process was carried out with chloroform.

Selection of buffer

Selection of buffer for extraction is an important step in the extraction method for the separation of aqueous and organic layer. It helps to separate two layers properly and increases the absorbance of drugs. We examined some of the buffers like phosphate buffer, acetate buffer and KCl-HCl buffer. Amongst these buffers, KCl-HCl buffer gave better separation and the dye-drug complex exhibited better intensity than in any other buffers. Hence, the experiments were carried out with the KCl-HCl buffer.

Effect of pH

The pH of KCl-HCl acidic buffer plays an important role in formation of ion-pair complex and intensity of color. The various values of pH were examined like pH-1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1 and 2.2. Out of all these, the KCl-HCl buffer of pH 2.2 gave better intensity of color for ion-pair complexes and, moreover, the complexes were found more stable in KCl-HCl buffer of pH 2.2. Hence KCl-HCl buffer of pH-2.2 was selected throughout the analysis.

Effect of dye concentrations

The dye concentrations were found by various trials. The BTB, MTR and MTO were used for OSP, ACL and FMV determination, respectively. Various concentrations like 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1% and 0.12% of all the dyes were examined. Amongst all these concentrations 0.1% gave acceptable intensity for all the dyes. The volume of dye solutions were varied from 0.1 mL to 2.0 mL and it was found that 1.0 mL of BTB, 0.5 mL of MTR and 2.0 mL of MTO gave satisfactory results for the corresponding drugs.

Effect of shaking time

The extraction process is based on the manual shaking by using the separating flask. The various shaking times were examined at 0.5, 1.0, 1.5 and 2.0 min in all the three drugs for extraction. The good results were found at 2.0 minutes for each extraction. Hence 2.0 minutes of shaking time was maintained for each extraction throughout the analysis.

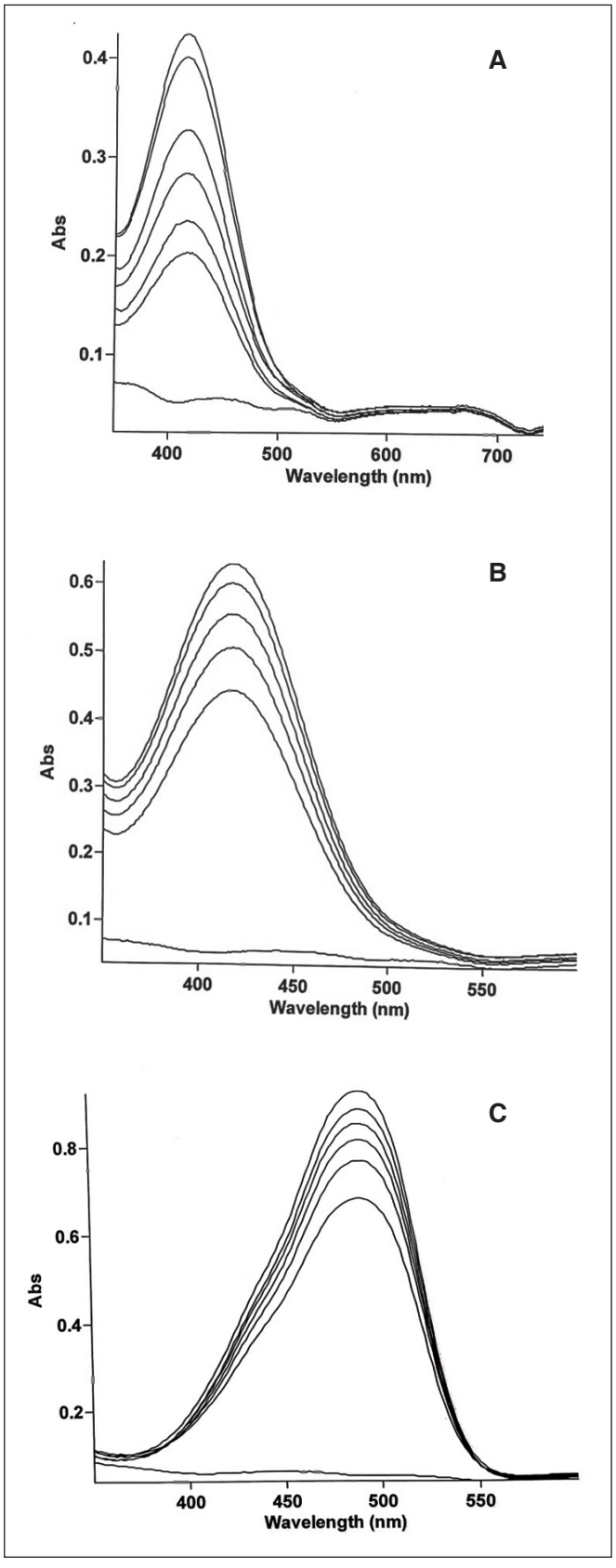


Fig. 2: Spectra of (A) OSP-BTP (B) ACL-MTR and (C) FMV-MTO complexes

METHOD VALIDATION

System suitability

The proposed method showed consistent absorbance at particular range for the respective drugs at 416 nm, 488 nm and 416 nm for OSP, ACL and FMV, respectively. Throughout the analysis, there was no significant divergence in absorbance. The percentage of relative standard deviations were found less than 2.0 % for the lowest concentration with six spikes of standards of OSP, ACL and FMV. The system suitability data are tabulated in Table II and typical spectra are given in Fig. 2.

Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ values showed that the proposed methods were found to have better sensitivity for all three drugs. The limit of detection values were found to be 0.5 µg mL^{-1}, 1.5 µg mL^{-1} and 1.0 µg mL^{-1} and limit of quantification values found were 1.7 µg mL^{-1}, 5.0 µg mL^{-1} and 3.3 µg mL^{-1} for OSP, ACL and FMV, correspondingly. The results are listed in Table II and were found to be satisfactory.

Linearity

For linearity, the spectra of standard solutions obtained at different concentrations of ion-pair complexes were recorded and plotted the graph of concentration of drug against absorbance. For OSP, ACL, and FMV, respectively, the linearity of the calibration graphs has been found to be between 1 and 5 µg mL^{-1}, 2 and 12 µg mL^{-1}, and 5 and 14 µg mL^{-1}. In this parameter, the color

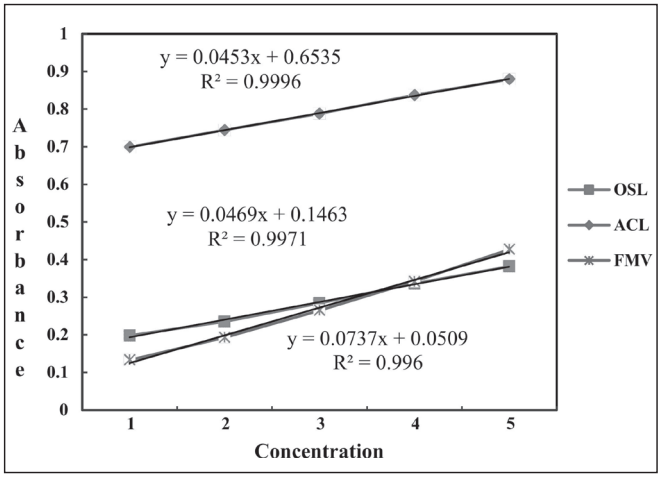


Fig. 3: Calibration curves for OSP, ACL and FMV

intensity of ion-pair complexes is directly proportional to the concentration of drug solution. The results are tabulated in Table II. The values of regression coefficients were found to be 0.996, 0.997 and 0.999 for OSP, ACL and FMV, respectively. The typical linearity graph is shown in Fig. 3.

Recovery

The accuracy parameter was confirmed by recovery studies. The analysis was carried out with the spiking of 60%, 80% and 120% analyzed against 100% concentrations of ion-pair complex solutions examined by UV spectrophotometer. The recovery values were found within the limit calculated according to standard procedure[11]. The results are shown in Table III.

Robustness

This parameter was assessed by looking at the ion-pair complex between drug-dye systems under optimal conditions using the suggested method. The various conditions like volume of the buffer (changed from 20 mL to 19 mL and 21 mL) and pH of the KCl-HCl buffer (from 2.2 to 2.1 and 2.3) were examined. For these variations, obtained outcomes are not diverging significantly. Hence the proposed method can be used at different conditions as well.

Ruggedness

The ruggedness of proposed method was examined by purposely changing the tentative environment such as different instruments, different manufacturers of solvents used for the method development. There were no significant deviations in outcomes throughout the analysis. These outcomes indicate that the proposed method is significantly rugged.

Specificity

Assay

The developed method use in the examination of formulations is its key feature. Tamiflu (for OSP), Tascort 400 (for ACL) and Virovir 500 (for FMV) were used. The samples equivalent to standard weight was weighed and added to the separating flask, followed by 20 mL of acidic KCl-HCl buffer, pH-2.2 and respective dye and chloroform. The mixture of the solution was shaken for 2 min for four times followed by extraction with chloroform. The spectra were interpreted and calculated according to standard formula. All the three drugs OSP, ACL and FMV gave good results (Table IV).

CONCLUSION

The contemporary methods for the assay of drugs by employing sophisticated instruments like HPLC, GLC, UPLC and MS offer good specificity, excellent precision and accuracy. But they include sophisticated equipment, which are not in the reach of most research laboratories and small-scale manufacturing industries. In the proposed method, ion-pair complex was formed by the electrostatic interaction between the selected drugs and corresponding dye. The mechanism takes place between the acidic group of dye and amine group of drug. The established method is simple, accurate and cost effective. All outcome parameters were found to be acceptable and all the three drugs were determined successfully in their pure and formulation forms without any interference in results. Hence it can be adopted in small-scale industries, research laboratories, and formulation industries for the determination of OSP, ACL and FMV in pure and their dosage form.

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