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Synthesis, Biological Evaluation and Validation Studies of Novel 5-(Substituted Aldehyde)-2-imino-7-methyl-3-oxo-N-phenyl-1,2,3,5-tetrahydroimidazo[1,2-a]pyrimidine-6-carboxamide Scaffolds

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Abstract

A series of 5-(substituted aldehyde)-2-imino-7-methyl-3-oxo-N-phenyl-1,2,3,5-tetrahydroimidazo [1,2-a]pyrimidine-6-carboxamide derivatives was synthesized and their chemical structures were confirmed by ^{1}H-NMR, FT-IR and elemental analysis studies. The synthesized derivatives were evaluated for their *in vitro* antimicrobial potential and validated by UV visible spectroscopy at absorbance 272 nm and comparable with lamivudine as standard. These compounds validated as per International Conference on Harmonization (ICH) guideline in the range of 2-20 µg/ml. Validation study indicated that, compounds 1, 2 and 3 exhibited good percentage purity at absorbance 272 nm and there is no interference of diluents at 272 nm. Antimicrobial results indicated that compound 2 was found to be most active against microbial species (bacterial and fungal).

Keywords: Validation; Pyrimidine; Precision; Accuracy; UV spectroscopy

Introduction

Heterocyclic compounds are widely used in medicinal field due to their structural variety. There are a large number of heterocyclic compounds i.e., pyrrole, morpholin, pyrrolidine, thiazole, pyran, thiophene, pyridine, pyrimidine and oxadiazole etc. play an important role in biological application. Pyrimidines having diverse biological and clinical applications and play wide role in drug discovery process [1]. This created interest among researchers who have synthesized variety of pyrimidine derivatives and screened them for their various biological activities i.e., antimicrobial, antioxidant, anticancer activity [2]. Pyrimidine is a heterocyclic moiety having six membered unsaturated ring system composed with carbon and two nitrogen molecules at 1 and 3 position of the pyrimidine ring [3]. Most abundant pyrimidine is uracil, cytosine and thymine. DNA and RNA bases are the most commonly identified pyrimidine bases and due to the component of nucleic acid, pyrimidine occupies the unique position in medicinal chemistry [4]. Pyrimidine is used as parent substance for the synthesis of a wide variety of heterocyclic compounds and raw material for drug synthesis [5].

Pharmaceutical analytical chemists have to face many problems and examine the number of impurities during organic synthesis at laboratory and raw material manufacturing. Therefore, various analytical techniques allow the satisfactory identification and quantification of target impurities by various methods [6]. Validation is a testing method is useful and necessary to access the applicability and possible limitation of method applied and to enhance the knowledge of analyst attention limits and on specificity of the method. Validation study has been done by the various analytical techniques such as UV, HPLC, reverse phase HPLC, HPTLC, UPLC, FTIR and Mass spectrometry for the various synthesized and plants extracted compounds [7].

In the validation various parameters are used i.e., accuracy, precision, linearity, specificity, robustness, LOQ (limit of quantification), stability, LOD (limit of detection) and ruggedness [8]. The purpose of stability testing is to grant facts on how the quality of a drug substance varies with time under the influence of a variety of environmental factors *viz.* light, temperature, humidity and to create a retest period for the drug substance and recommended storage conditions. The main goal of broadcasting is identify and detect the existence of pollutants in the sample of the compound, qualitative validation practice establishing the minimum concentration for each compound that can be identified (limit of identification) and detected (screening detection limit) in a reliable way in the different prevailing conditions under study [9]. On the basis of literature survey, we may conclude that all the synthesized compounds in laboratory should be validated by specific technique or which give better purity of compounds.

Materials and Methods

Chemistry

In the present work, we report a simple synthetic method for the synthesis of new series (Scheme 1) of pyrimidine derivatives by the reaction of acetoacetanilide and substituted aldehyde with guanidine nitrate in the presences of $AlCl_3$, HCl and CH_3OH yielded intermediate-1. The intermediate-1 reacts with dextrose compound in the presences of monochloroacetic acid, ethyl acetoacetate and sodium benzoate yielded final pyrimidine derivatives (1-3). The chemical structures of the synthesized compounds were confirmed by ^{1}H-NMR, FT-IR spectral data and elemental analysis. The validation study of the synthesized compounds was carried out by UV-VIS spectroscopy technique. The physicochemical properties and chemical structure of compounds are presented in Table 1.

Experimental Section

Synthesis of pyrimidine derivatives followed the general procedure discussed in synthetic Scheme 1. All reagents and solvents used in study without any further purification in laboratory. Reaction steps forward was observed by thin layer chromatography (TLC) making use of commercial silica gel plates (Merck), Silica gel F254 on aluminium

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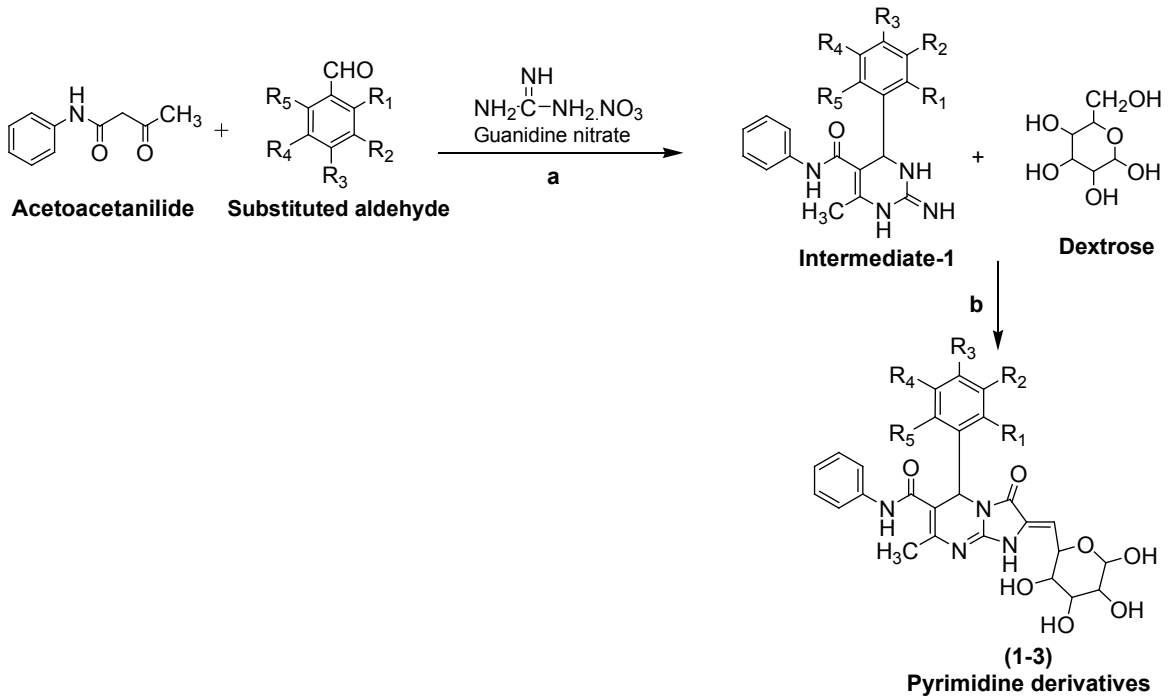


Where **a**): $AlCl_3$, HCl, CH_3OH, Reflux 11-12 hrs; **b**): Monochloroacetic acid, Ethyl acetoacetate, Sodium benzoate, Glacial acetic acid, Reflux 6-7 hrs

Compounds	R_1	R_2	R_3	R_4	R_5
1.	H	OCH_3	OCH_3	OCH_3	H
2.	OH	H	H	Br	H
3.	H	Cl	H	H	H

Scheme 1: For the synthesis of 5-(substituted aldehyde)-2-imino-7-methyl-3-oxo-*N*-phenyl-1,2,3,5-tetrahydroimidazo [1,2-*a*]pyrimidine-6-carboxamide scaffolds.

sheets. Melting points were tested in open capillary tubes method. ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra data recorded by Bruker Avance 400 NMR spectrometer in appropriate DMSO-deuterated solvents and are expressed in parts per million (δ, ppm) downfield from tetramethyl silane (internal standard). ^{1}H- NMR data are given as multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet) and number of protons. Infrared (IR) spectra were recorded on Bruker 12060280, Software: OPUS 7.2.139.1294 spectrometer. The elemental analysis results of synthesized compounds were within ± 0.3% of the theoretical values.

Procedure for the synthesized derivatives (1-3)

Step 1: The synthesis of Intermediate-1 (2-imino-6-methyl-4-(substituted aldehyde)-N-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamide): A mixture of substituted aldehyde (0.01 mol), guanidine nitrate (0.015 mol) and acetoacetanilide (0.01 mol) were placed in round bottom flask in methanol (50 ml) and added aluminum chloride (0.003 mol) with 2-3 drops of conc. hydrochloric acid as catalyst amount then the reaction mixture was refluxed for 11-12 hrs after that the reaction mixture was cooled to room temperature and poured into crushed ice water with vigorous stirring, filtered and recrystallized with methanol [10].

Step 2: The synthesis of 5-(substituted aldehyde)-7-methyl-3-oxo-N-phenyl-2-((3,4,5,6-tetrahydroxy-tetrahydro-2H-pyran-2-yl)methylene)-1,2,3,5-tetrahydroimidazo[1,2-a] pyrimidine-6-carboxamide derivatives: A mixture of intermediate-1 (0.01 mol), sodium benzoate (2 g), dextrose (0.01 mol), glacial acetic acid (20 ml), ethyl acetoacetate (15 ml) and monochloroacetic acid (0.015 mol) were taken in RBF and refluxed with controlled temperature at 140-142°C for 6-7 hrs. The reaction mixture was cooled at room temperature and poured into ice cold water to yielded solid precipitate or titled compounds (1-3), filtered and recrystallized with methanol.

Spectral data

Compound 1 (7-Methyl-3-oxo-N-phenyl-2-((3,4,5,6-tetrahydroxy-tetrahydro-2H-pyran-2-yl)methylene)-5-(3,4,5-trimethoxyphenyl)-1,2,3,5-tetrahydroimidazo[1,2-a]pyrimidine-6-carboxamide): IR (KBr pellets, cm^{-1}): 3062 (C-H str., phenyl nucleus), 1596 (C=C str., phenyl nucleus), 694 (C-C str., phenyl nucleus), 1630 (C=O str.,), 3321 (N-H str., 2˚amide), 1630 (N=CH str., pyrimidine), 1244 (C-N str., pyrimidine), 2779 (C-H str., cyclic ether), 1126 (C-O-C str., aryl ether), 3321 (O-H str., polyhydroxy on dextrose), 1244 (C-O-C str., OCH_3); ^{1}H NMR (DMSO-d_6, δppm): 7.45-7.49 (m, 7H, Ar-H), 2.10 (s, 1H, NH, amide), 8.25 (s, 1H, NH, amide), 3.86-4.22 (m, 5H, CH, tetrahydropyran), 2.10 (m, 4H, OH alcohol), 3.86 (m, 9H, OCH_3).

Compound 2 (5-(5-Bromo-2-hydroxyphenyl)-7-methyl-

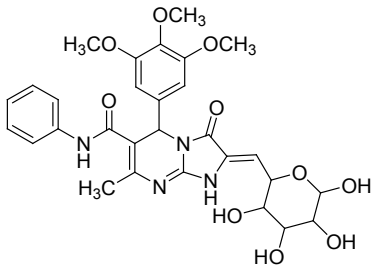
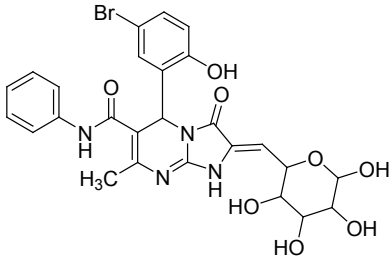
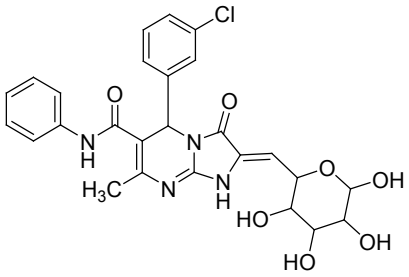
Compounds	Structure	M. Formula	M. Wt.	m.p. (°C)	R_f Value*	% Yield	*TLC mobile phase
1.		$C_{29}H_{32}N_4O_{10}$	596	169-171	0.41	70.04	Benzene
2.		$C_{26}H_{25}BrN_4O_8$	601	119-121	0.52	85.55	Benzene
3.		$C_{26}H_{25}ClN_4O_7$	540	150-153	0.45	65.34	Benzene

Table 1: The physicochemical properties and chemical structure of compounds.

3-oxo-N-phenyl-2-((3,4,5,6-tetrahydroxy-tetrahydro-2H-pyran-2-yl)methylene)-1,2,3,5-tetrahydroimidazo[1,2-a] pyrimidine-6-carboxamide): IR (KBr pellets, cm^{-1}): 3062 (C-H str., phenyl nucleus), 1596 (C=C str., phenyl nucleus), 691 (C-C str., phenyl nucleus), 1631 (C=O str., 2°amide), 1712 (C=O str., aryl ketone), 3332 (N-H str., 2° amide), 1631 (N=CH str., pyrimidine), 1282 (C-N str., pyrimidine), 2832 (C-H str., cyclic ether), 1070 (C-O-C str., aryl ether), 3332 (O-H str., polyhydroxy on dextrose), 3332 (OH str., phenyl), 543 (C-Br str., phenyl nucleus); ^{1}H-NMR (DMSO-d_6, δppm): 6.66-7.63 (m, 8H, Ar-H), 1.98 (s, 1H, NH, amide), 8.11 (s, 1H, NH, amide), 1.75 (s, 1H, CH_3), 6.66 (s, 1H, ethylene), 3.79-5.12 (m, 5H, CH, tetrahydropyran), 1.98 (m, 4H, OH alcohol), 5.07 (s, 1H, OH).

Compound 3 (5-(3-Chlorophenyl)-7-methyl-3-oxo-N-phenyl-2-((3,4,5,6-tetrahydroxy-tetrahydro-2H-pyran-2-yl)methylene)-1,2,3,5-tetrahydroimidazo[1,2-a]pyrimidine-6-carboxamide): IR (KBr pellets, cm^{-1}): 3057 (C-H str., phenyl nucleus), 1596 (C=C str., phenyl nucleus), 689 (C-C str., phenyl nucleus), 1666 (C=O str., 2° amide), 1717 (C=O str., aryl ketone), 3327 (N-H str., 2° amide), 1666 (N=CH str., pyrimidine), 1315 (C-N str., pyrimidine), 2830 (C-H str., cyclic ether), 1082 (C-O-C str., aryl ether), 3327 (O-H str., polyhydroxy on dextrose), 758 (C-Cl str.); ^{1}H-NMR (DMSO-d_6, δppm): 7.28-7.65 (m, 9H, Ar-H), 1.97 (s, 1H, NH, amide), 8.11 (s, 1H, NH, amide), 1.85 (s, 1H, CH_3), 3.47-5.00 (m, 5H, CH, tetrahydropyran), 1.97 (m, 4H, OH alcohol).

Biological Section

Antimicrobial activity

The *in vitro* antimicrobial potential of synthesized compounds against Gram-positive and Gram-negative bacterial and fungal species was demonstrated by tube dilution technique. Norfloxacin and fluconazole used as standard for antibacterial and antifungal activities respectively. Dilutions of synthesized compounds and standard were prepared in double strength nutrient broth for bacterial strains and sabouraud dextrose broth for fungal strains. The samples were incubated at 37 ± 1°C for 24 h (bacteria), at 25 ± 1°C for 7 days (*A. niger*) and at 37 ± 1°C for 48 h (*C. albicans*) respectively and the results were recorded in terms of MIC (the lowest concentration of test substance which inhibited the growth of microorganisms). Antimicrobial results indicated that in case of Gram positive bacteria, compounds 1 (MIC_{sa}=3.11 µM/ml) showed significant activity against *S. aureus* and compound 3 (MIC_{bs}=0.48 µM/ml) exhibited most potent against *B. subtilis*. In case of Gram negative bacterium, compound 2 (MIC_{ec}=2.06 µM/ml) displayed more potent against *E. coli* and antifungal activity ($MIC_{ca\ \&\ an}$=1.03 µM/ml) was found to be most potent against *C. albicans* and *A. niger* and may be used as lead to development novel antimicrobial agents. Results of antimicrobial potential presented in Table 2.

Validation Section

Method

Validation is defined as establishing recognized evidence, which give a high grade of assurance that a specific activity will consistently produce a desired result or product gathering its predetermined specifications and quality characteristics. Lamivudine was taken as standard for validation study of most potent antimicrobial compounds 1, 2 and 3 and were selected for validation study [11].

Determination of λ_{max}

Prepared standard stock solution 100 µg/ml by dissolved an accurately weighed 5 mg lamivudine drug in to 50 ml ethanol. Then 1 ml of standard stock solution was transferred into 10 ml volumetric flask and makeup the volume up to mark to prepare working standard solution 10 µg/ml. This solution was scanned within UV-VIS wavelength of 200-400 nm against solvent blank used as reference. Result of standard drugs showing in spectrum with 272 nm (λ_{max}) wavelength (Figure 1), this wavelength used for further analysis of newly synthesized derivatives [12].

Preparation solution of synthesized compound/standard drug

An accurately weighed 5 mg of synthesized compound/standard was transferred into 50 ml volumetric flask and 50 ml of ethanol was added, dissolved it and make up the volume up to mark with ethanol. This solution was filtered through whatman filter paper and obtained final conc. 10 µg/ml take 1 ml of this filtrate was diluted to 10 ml with ethanol and measured absorbance at 272 nm against solvent blank. Percentage (%) assay was calculated from using equation 1 and from it calculates the mean value, standard deviation and percentage RSD (Relative Standard Deviation).

$$\% \text{ Assay} = \frac{\text{Abs. of test compound}}{\text{Abs.of standard}} \times \frac{\text{Wt of std}}{\text{Dil. factor}} \times \frac{\text{Dil factor}}{\text{Wt of test compound}} \times 100 \quad (1)$$

Precision

The precision of an analytical practice express the proximity of agreement (degree of scatter) between a sequences of measurement obtained from many sampling of the same homogeneous sample under the prescribed conditions. Precision is frequently expressed as standard deviation and % RSD.

Different analysts

The synthesized compounds were analyzed using proposed method by three different analysts and results are presented in Table 3.

Compound	Minimum Inhibitory Concentration (MIC)				
	Bacterial species			Fungal species	
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*	*A. niger*
1.	3.11	2.10	2.10	1.06	1.07
2.	4.16	1.04	2.06	1.03	1.03
3.	4.63	0.48	2.31	2.33	2.31
Std.	0.47[a]	0.47[a]	0.47[a]	0.50[b]	0.50[b]

[a]Norfloxacin; [b]Fluconazole

Table 2: Antimicrobial potential (MIC=µM/ml).

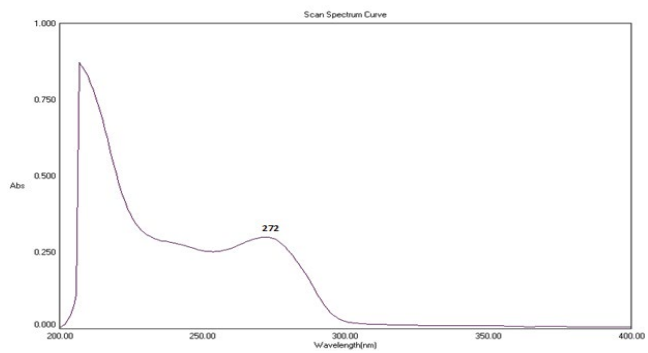


Figure 1: λ_{max} value of Standard drug (lamivudine).

Different instruments

The synthesized compounds were analyzed using proposed method by two different instruments to check instrument to instrument variation (Table 4).

Different days

The synthesized compounds (1-3) were analyzed using proposed method by three different days and results are presented in Table 5.

Linearity range

The linearity of an analytical method is its capability to obtain test results within a given range which are directly proportional to the concentration of analyte in the test sample. Prepared a series of concentrations of synthesized compound/standard ranging from 2-20 µg/ml separately and plot the graphs (Figures 2-5) of conc. v/s absorbance were found to be a straight line (Table 6).

Robustness

The robustness of an analytical method is determined of its capacity to remain unaffected by small, but premeditated variations in method parameters and affords an indication of its reliability during normal usage. The synthesized compound /standard were analyzed using

Comp.	% Assay by different analysts*			Mean	± S.D.	% RSD
	I	II	III			
1.	101.52	99.28	100.95	100.58	1.1641	1.1574
2.	99.59	99.97	100.89	100.15	0.6684	0.6674
3.	100.32	100.19	98.68	99.73	0.9116	0.9141

*Each value is mean of five observations

Table 3: Precision (different analysts).

Comp.	% Assay by different instruments*		Mean	± S.D.	% RSD
	UV-I (UV-1800)	UV-II (UV-3000+)			
1.	99.19	99.53	99.36	0.2404	0.2420
2.	100.31	99.87	100.09	0.3111	0.3108
3.	99.57	99.67	99.62	0.0707	0.0710

*Each value is mean of five observations

Table 4: Precision (different instruments).

Comp.	% Assay by different days*		Mean	± S.D.	% RSD
	Interday	Intraday			
1.	99.74	99.46	99.60	0.1980	0.1988
2.	100.63	100.59	100.61	0.0283	0.0281
3.	99.90	99.96	99.93	0.0424	0.0425

*Each value is mean of five observations

Table 5: Precision (different days).

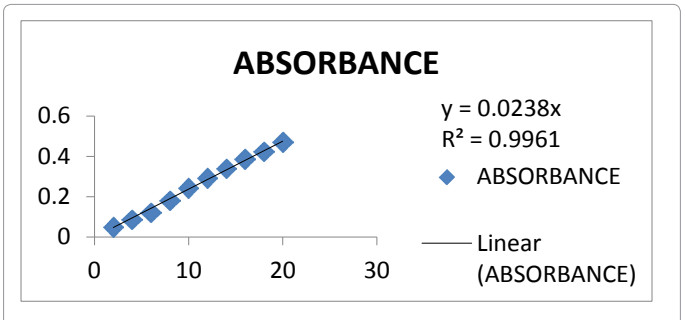


Figure 2: Linearity curve of standard drug (lamuvudine).

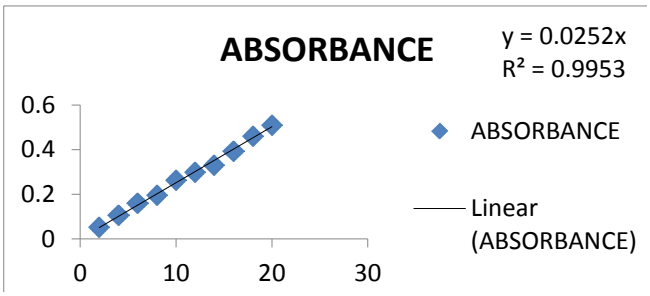


Figure 3: Linearity curve of compound 1.

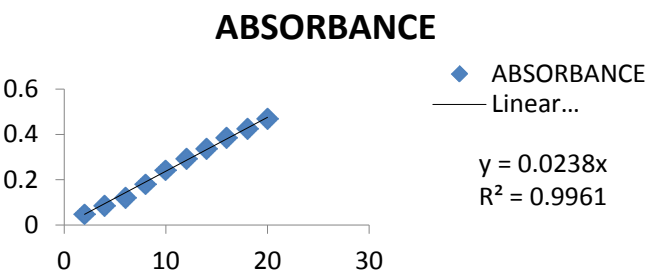


Figure 4: Linearity curve of compound 2.

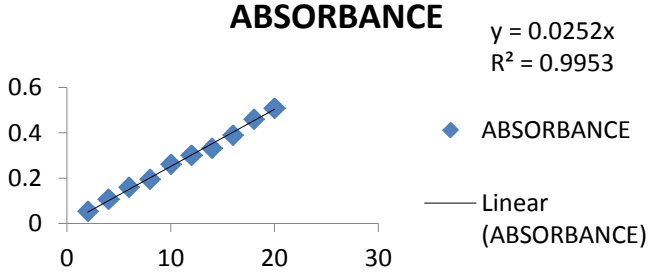


Figure 5: Linearity curve of compound 3.

proposed method at different wavelength i.e., 270 nm, 272 nm and 274 nm. The results are showing in Table 6.

Specificity

Specificity is the capability to assess unequivocally the analyte in the presence of components which may be estimated to be present. Usually these may be including matrix, impurities, degradants etc. An accurately weighed six different quantities of 5 mg of synthesized compound /standard was transferred into 50 ml volumetric flasks and stored for 24 hrs under different conditions given below:

- At 50°C after addition of 1 ml of 0.1 N NaOH solution.
- At 50°C after addition of 1 ml of 0.1 N HCl solution.
- At 50°C after addition of 1 ml of 3% H_2O_2
- Heat at 60°C
- In UV chamber
- Normal

After 24 h, ethanol was added in each flask and sonicated for 10 min and make up the volume with ethanol, 1 ml solution was transferred from each flask in different 10 ml volumetric flask and adjusts the volume with ethanol to obtain the concentration of 10 µg/ml and measured the absorbance at 272 nm against blank (ethanol). The results are presented in Table 7.

Accuracy

Accuracy of the proposed method was expresses the nearness of agreement between the value which is received either as a predictable true value or an accepted reference value and the value found.

An accurately weighed 10 mg of synthesized compound was transferred into 100 ml volumetric flask and to it standard drug (lamivudine) was added so as 80%, 100% and 120% respectively of total test compound and then 50 ml of ethanol was added in each flask, sonicated for 10 min. Volume was make up with ethanol and each solution was filter through whatman filter paper. 1 ml of each filtrate was diluted up to 10 ml with ethanol and measured the absorbance at 272 nm against blank. The percentage (%) recovery was calculated by using equation 2. The presented results are showing in Tables 8 and 9.

$$\% \text{ Recovry} = \frac{X}{Y+Z} \times 100 \quad (2)$$

Where,

X – Total drug estimated

Y – Weight of test compound

Z – Amount of std. drug added

Conclusion

Validation study as per ICH guidelines, we may conclude that compounds (1-3) exhibited good percentage purity when compared with standard drug lamivudine at absorbance 272 nm. The method was appropriate for precise, accurate, specific, linear, robust, having stability determination of characteristics and simple, sensitive and cost effective, hence can be used for routine analysis for intended purpose of newly synthesized derivative. From the biological potential, compound 2 exhibited significant antimicrobial activity against microbial species and comparable with standard drugs and may be used to development novel category of the antimicrobial agents.

Compound	Coefficient of correlation	Slope	Range (µg/ml)
1.	0.995	0.023	2-20
2.	0.996	0.023	2-20
3.	0.995	0.025	2-20
Lamuvudine	0.997	0.026	2-20

Table 6: Linearity range of standard and tested compounds.

Comp.	% Assay at different wavelength*			Mean	± S.D.	% RSD
	270 nm	272 nm	274 nm			
1.	98.83	99.43	97.83	98.70	0.8083	0.8190
2.	99.04	100.07	99.33	99.48	0.5311	0.5339
3.	99.49	99.89	99.27	99.55	0.3143	0.3157

*Each value is mean of five observations

Table 7: Robustness.

Comp.	% Assay under different conditions*					
	Normal	0.1N HCl	0.1N NaOH	3% Peroxide	60°C Heat	UV Chamber
1	101.40	94.66	94.01	102.20	101.87	104.43
2	100.97	95.98	94.55	101.32	100.72	103.77
3	99.72	94.92	93.52	101.41	99.92	103.83

*Each value is mean of five observations

Table 8: Specificity.

Comp.	% Recovery*			Mean	± S.D.	% RSD
	(Sample weight+Std drug added) in mg					
	10+8	10+10	10+12			
1	98.87	98.96	100.03	99.29	0.6453	0.6500
2	100.14	100.07	99.88	100.03	0.1345	0.1345
3	99.13	99.56	100.81	99.83	0.8727	0.8742

*Each value is mean of five observations

Table 9: Results of accuracy.

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