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Research Article

STABILITY INDICATING SPECTROPHOTOMETRIC METHODS OF NELFINAVIR AND THEIR APPLICATION TO *IN-VITRO* BIOEQUIVALENCE TESTING

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ABSTRACT
The present investigation was aimed at developing the stability indicating spectrophotometric methods for the determination of nelfinavir mesylate (NEM) in pharmaceutical dosage forms. The stability of NEM was tested in various dissolution media maintained at ambient temperature and 37°C for 48 h. Stability studies of NEM in various media indicated that the drug was stable in 0.1M HCl and pH 7.8 phosphate buffer. The λ_{max} were found 201.4 and 212.0 nm for 0.1M HCl and pH 7.8 phosphate buffers respectively with low coefficient of variation of < 5.11 %. The linearity of NEM was found in the range of 0.5 – 60 µg/mL for 0.1M HCl and 0.5 – 40 µg/mL for pH 7.8 phosphate buffer. The validated methods were applied to determine NEM concentration in formulations. *In-vitro* dissolution testing indicated that the NEM was stable and drug release was uniform from tablet dosage forms. The optimized media could be employed to study the dissolution profiles of NEM in bioequivalence studies.
Keywords: Nelfinavir mesylate, stability indicating, bioequivalence studies, dissolution media.

INTRODUCTION

Dissolution testing is based on the fact that the drug enters into solution per unit time. *In vitro* dissolution test is a quality control parameter to check batch-to-batch consistency of drug release from a formulation. It plays a significant role to assess the possible *in vivo* bio availability based on *in vitro in vivo* correlation of drug products[1]. Development of dissolution medium is one of the critical factors to perform dissolution test for any drug. It is often sought-after to have a dissolution medium that is stable and selective based on the formulation used. Dissolution media were developed earlier to improve the solubility of lipophilic drugs such as nimodipine[2], cefixime trihydrate[3], rifampicin[4] and valdecoxib[5] in dissolution media. The US FDA's recent guidance has increased awareness among industries and scientists regarding dissolution testing[6-8]. The FDA has not only provided guidelines for dissolution tests for oral modified release dosage forms, but also pointed to the need for individualizing the methods on a case by case for the drug products. The generic forms of many drugs are being formulated as extended release products after the expiry of patents. The authorized USP pending monograph for nelfinavir mesylate tablets specifies 0.1N HCl as dissolution medium[9]. We have earlier reported stability indicating dissolution media of abacavir sulphate did anosine and lamivudine and proposed their application for extended release formulations[10-12]. Therefore there is a tremendous scope for pharmaceutical scientists to develop suitable dissolution testing media for bioequivalence studies of newly developed formulations. Nelfinavir mesylate is 2-[2-hydroxy-3-(3-hydroxy-2-methyl-benzoyl)amino-4-phenylsulfanyl-butyl]-N-tert-butyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxamide (Figure 1). Nelfinavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Nelfinavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral poly proteins resulting in the formation of immature non-infectious viral particles. Nelfinavir inhibits the HIV viral proteinase enzyme which prevents cleavage of the gag-pol poly protein, resulting in noninfectious, immature viral particles[13,14]. Nelfinavir mesylate (NEM) was selected as drug in the present study because more varieties of generic formulations are coming up in the market both as conventional and extended release dosage forms. The present investigation is aimed at designing the stability indicating dissolution media for the determination of nelfinavir mesylate in pharmaceutical dosage forms such as conventional and extended release tablets in bioequivalence studies.

MATERIALS AND METHODS

Materials

Pure nelfinavir mesylate (NEM) was obtained as gratis sample from Matrix Laboratories, Hyderabad, India. Potassium dihydrogen phosphate of AR grade and other chemicals of AR grade were purchased from E. Merck® (India) Ltd., Mumbai, India. Water used was triple distilled grade and prepared by all glass distillation apparatus (quartz distillation unit, Borosil®). Three brands of nelfinavir mesylate 250 mg tablets, i.e., Neve (P), Nevimune (Q) and Neviretro (R) were purchased from local market.

Stability studies

Nine dissolution media were selected and prepared such as distilled water, 0.1M HCl, pH 1.2 KCl-HCl buffers and pH 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8 phosphate buffers as per the standards of United States Pharmacopoeia[15]. The pH of the buffers was measured and adjusted using pH Analyzer (Elico®, Model No. LI612). Stock solutions of NEM were prepared by dissolving accurately weighed (Afcoset® electronic balance) 25 mg of NEM in 2 mL of methanol and the volume was made up to 25 mL of distilled water, 0.1M HCl, pH 1.2 KCl-HCl buffer and pH 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8 phosphate buffer separately to obtain 1 mg/mL solutions. All the solutions were sonicated using an ultrasonic bath (Enertech®) to dissolve the drug. From these solutions

2.5 mL was pipette out (Genie® micropipettes) into 25 mL volumetric flask and diluted with the same solvent system to obtain 100 µg/mL solutions. The stability of 100 µg/mL solutions of NEM was tested in the above prepared dissolution media at room temperature (RT, 25 ± 5°C) and 37 ± 2°C in an incubator (Thermolab®) for 48 h separately. Two sets of these solutions were prepared and maintained at RT and 37°C in an incubator. All the samples were centrifuged (Remi®) for 5 minutes before scanning. The samples were scanned at 0, 24 and 48 h intervals using a double-beam UV-visible spectrophotometer (Elico®, India, model SL 169) connected to computer loaded with Spectral Treats® software. The λ_{max} and absorbance were measured to verify any deviations in the values. The above procedure was followed for all the media. The dissolution media that have shown stability of the drug were selected for further evaluation.

Development and validation of analytical methods

Standard graphs of NEM were constructed for the selected dissolution media after optimizing the conditions based on stability studies. Absorbance's were determined for NEM at selected λ_{max} values using UV-visible spectrophotometer (Elico®, India, model SL 169) for each of the above selected stable media after making dilutions to obtain 0.1 – 100 µg/mL concentrations from the stock solutions. The beer's limit was determined from the constructed plots of wavelength vs absorbance. The proposed methods were validated for accuracy, precision and robustness. The methods were tested for intra- and inter-day variations. The recovery studies were carried out by adding known amounts of (10 µg and 20 µg) of NEM to the pre-analyzed samples and subjecting them to the proposed UV spectrophotometric methods. Replicates of six samples were tested in the above studies.

Assay of nelfinavir mesylate in commercial formulations

The estimation of NEM content in commercial formulations was carried out in the developed analytical methods using selected dissolution media. Contents of ten tablets containing NEM were pooled and powdered. The powder equivalent to 25 mg of NEM was extracted into selected medium and the volume was adjusted to 25 mL, mixed by sonication and filtered through a 0.45 µm Whatman filter paper. From the filtrate 0.1 mL was pipette into a 10 mL graduated test tube and then the volume was adjusted to 10 mL with the dissolution medium and was assayed for NEM content using selected methods. The above procedure was followed for remaining tablet brands and for all the selected methods in replicates of six.

***In-vitro* dissolution rate testing**

The *in-vitro* test was conducted to verify the stability of NEM in the optimized and selected dissolution media during the dissolution testing. The dissolution testing was carried out in a six-stage dissolution rate testing apparatus USP XXI (Labindia, Mumbai, India). A 900 mL of the selected dissolution medium was taken separately and dissolution test was performed using the paddle method at 37°c and 75 RPM. Aliquot volumes of 5 mL each were withdrawn from the dissolution bowl at various time intervals, i.e., 5, 10, 15, 20, 30, 35, 45 and 60 minutes. The samples were replaced by an equal volume of media and analyzed at selected λ_{max} using UV-visible spectrophotometer (Elico®, India, model SL 169) against blank solution containing dissolution medium after suitable dilution. The dissolution test was performed on the tablets of brand P employing the selected dissolution media.

RESULTS AND DISCUSSION

The stability of 100 µg/mL solutions of nelfinavir mesylate was successfully tested in nine dissolution media such as distilled water, 0.1M HCl, pH 1.2 KCl-HCl buffer and pH 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8 phosphate buffers as per the standards of IP maintained at RT and 37°C for 48 h separately. Stability studies of NEM in various media at RT and 37°C indicated that the drug was stable in 0.1M HCl and pH 7.8 phosphate buffer in the UV region for a period of 48 h. The results are summarized in Table 1 and Figures 2 and 3.

Table 1: Stability of NEM in various media at 37°C

Medium	0 h		24 h		48 h		% CV[a]
	λ_{max}	Absorbance	λ_{max}	Absorbance	λ_{max}	Absorbance	
Distilled Water	195.5 (± 0.1)	2.831 (± 0.014)	191.5 (± 0.1)	1.707 (± 0.008)	191.5 (± 0.1)	1.906 (± 0.016)	27.92
0.1M HCl	201.4 (± 0.1)	2.198 (± 0.008)	201.5 (± 0.1)	2.258 (± 0.009)	201.4 (± 0.1)	2.461 (± 0.011)	4.78
pH 1.2	207.5 (± 0.1)	0.154 (± 0.006)	203.4 (± 0.1)	0.503 (± 0.007)	207.4 (± 0.1)	0.500 (± 0.013)	52.02
pH 5.8	191.4 (± 0.1)	1.626 (± 0.005)	191.5 (± 0.1)	1.758 (± 0.012)	191.5 (± 0.1)	1.936 (± 0.021)	8.77
pH 6.2	193.0 (± 0.1)	1.390 (± 0.008)	193.4 (± 0.1)	0.643 (± 0.016)	193.4 (± 0.1)	1.510 (± 0.008)	39.77
pH 6.6	216.6 (± 0.1)	3.706 (± 0.003)	216.0 (± 0.1)	1.358 (± 0.007)	216.6 (± 0.1)	0.897 (± 0.006)	75.81
pH 7.0	214.4 (± 0.1)	0.273 (± 0.011)	214.3 (± 0.1)	2.346 (± 0.011)	212.4 (± 0.1)	1.186 (± 0.014)	81.91
pH 7.4	212.4 (± 0.1)	2.196 (± 0.014)	212.4 (± 0.1)	1.559 (± 0.014)	212.4 (± 0.1)	1.067 (± 0.009)	35.21
pH 7.8	212.0 (± 0.1)	1.720 (± 0.004)	212.0 (± 0.1)	1.697 (± 0.009)	212.2 (± 0.1)	1.863 (± 0.014)	5.11

Values in parenthesis are ±standard deviation (n = 6); [a] % CV = percent coefficient of variation of absorbance's of 0, 24 and 48 h

Table 2: Optical characteristics and regression analysis of proposed analytical methods in selected dissolution media

Parameter	Method A (0.1M HCl)	Method B (pH 7.8 buffer)
Optical characteristics:		
Beer's Law limit (µg/mL)	0.5–60	0.5–40
Sandell's sensitivity (µg/cm^2/0.001 absorbance unit)	0.041841	0.035631
Molar Extinction coefficient (1 mole^{-1}.cm^{-1})	1.36 x 10^6	1.18 x 10^6
Regression analysis:		
Slope (m)	0.025	0.034
Intercept (c)	0.001	0.0021
Standard error	0.014478	0.015326
Regression coefficient (r^2)	0.9993	0.9996

y = mx + c, where 'x' is concentration in µg/mL and 'y' is absorbance unit.

Table 3: Precision of the proposed methods

Method	Selected λ_{max} (nm)	NEM concentration (µg/mL)	Concentration of NEM (µg/mL) found on			
			Intra-day		Inter-day	
			Mean (n = 6)	% CV	Mean (n = 6)	% CV
A	201.4	10	9.98	1.59	10.67	2.13
		30	30.09	2.62	30.16	2.49
B	212.0	10	10.11	1.48	10.18	1.38
		30	30.15	2.19	30.19	1.17

% CV = percent coefficient of variation

Table 4: Recovery studies of NEM

Method	Selected λ_{max} (nm)	Amount of drug added (µg)	Mean (± S.D.) amount (µg) found (n=6)	Mean % recovery
A	201.4	10	9.967 (± 0.08)	99.96
		20	19.991 (± 0.13)	99.98
B	212.0	10	10.011 (± 0.07)	100.22
		20	20.013 (± 0.17)	100.26

Values in parenthesis are ± standard deviation (n = 6)

Table 5: Assay of different brands of NEM tablets

Method	Brand	Labeled amount of drug (mg)	Mean % of labeled amount (n = 6)	% CV
A	P	250	97.64	2.24
	Q	250	102.15	2.46
	R	250	100.29	1.73
B	P	250	101.88	1.68
	Q	250	100.27	2.07
	R	250	99.87	1.13

% CV = percent coefficient of variation

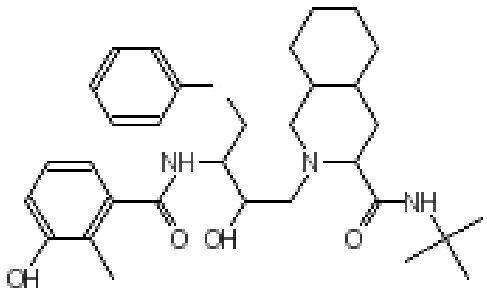

Figure 1: Structure of nelfinavir mesylate

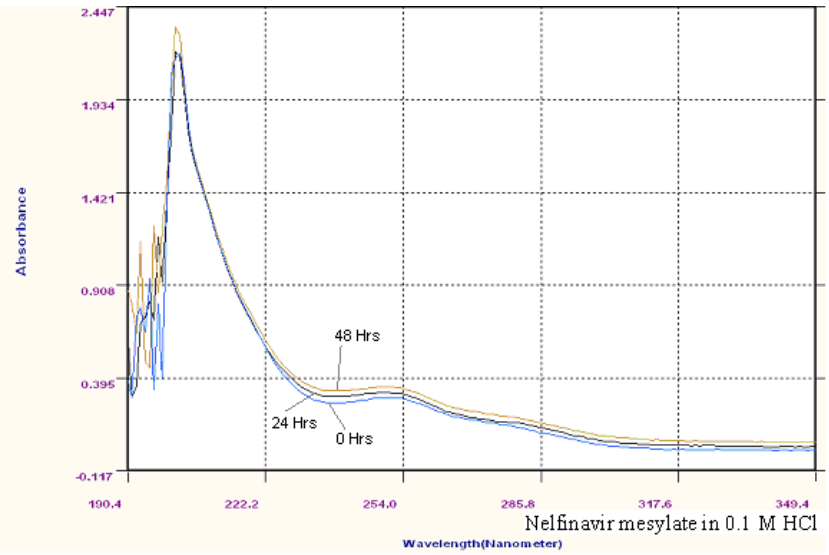

Figure 2: Scanning curves of NEM in 0.1 M HCl at time intervals of 0, 24 and 48 h and temperature 37°C

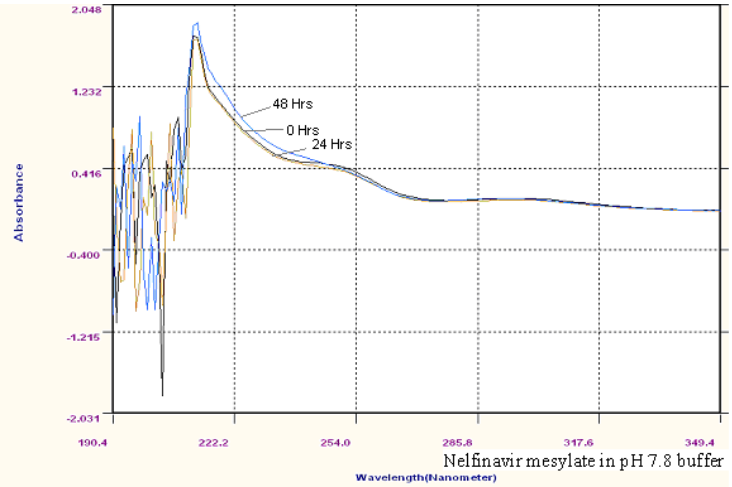

Figure 3: Scanning curves of NEM in pH 7.8 phosphate buffers at time intervals of 0, 24 and 48 h and temperature 37°C

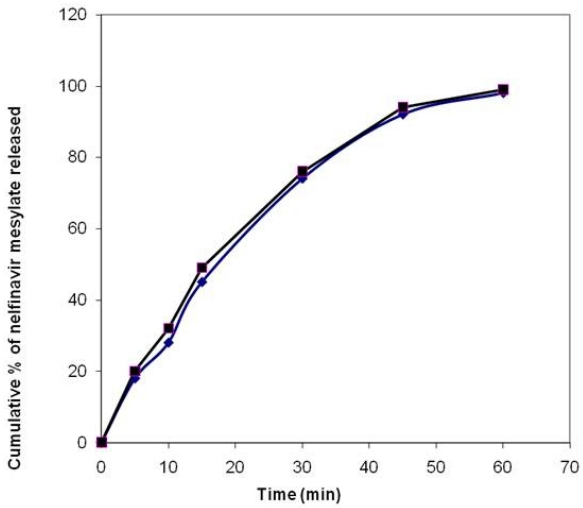

Figure 4: Cumulative % of NEM (brand P) released vs time plots in 0.1M HCl (–♦–) and pH 7.8 phosphate buffers (–■–)

The λ_{max} were found 201.4 and 212.0 nm for 0.1M HCl and pH 7.8 phosphate buffers respectively with an observed low coefficient of variation of < 5.11 %. The drug was unstable in other dissolution media and hence 0.1M HCl and pH 7.8 phosphate buffer were selected as dissolution media to estimate NEM in formulations. The analytical methods for NEM in the two selected dissolution media were developed and named as method A and B for 0.1M HCl and pH 7.8 phosphate buffers respectively. Standard graphs were constructed and linearity of the graphs were found in the range of 0.5 – 60 µg/mL and 0.5 – 40 µg/mL for 0.1M HCl and pH 7.8 phosphate buffer respectively. The regression equations from calibration graphs were found: $y = 0.025x – 0.001$ ($r^2 = 0.9993$) and $y = 0.001x + 0.0021$ ($r^2 = 0.9996$) for media stated in the above order. The optical characteristics and regression analysis of proposed analytical methods in selected dissolution media are summarized in Table 2. The methods were validated for precision, accuracy and robustness. A low coefficient of intra- and inter-day variation of 1.17–2.48% indicated that the two methods were highly precise (Table 3). About 99.94, 99.98, 100.26 and 100.28 % of NEM could be recovered from the pre analyzed samples using these methods indicating that the proposed methods were accurate (Table 4). Assay of three brands (P, Q and R) of NEM was determined using the developed methods. The mean amount of NEM determined to be 97.64 (102.15 %) and 99.87 (101.88 %) of the labeled amount for methods A and B respectively (Table 5). The low percent of coefficient of variation (1.13 – 2.46 %) indicated that the reproducibility of the assays of NEM in the tablet dosage forms. The commonly used excipients and additives in the pharmaceutical formulations did not interfere with the proposed methods. The *in-vitro* dissolution test was conducted to verify the stability of NEM in the optimized and selected dissolution media during the dissolution testing. The dissolution test was performed on the tablets of brand P employing the two dissolution media which were optimized. The results of *in-vitro* dissolution testing indicated that the drug was stable in simulated gastric fluid (0.1M HCl) and simulated intestinal fluid (pH 7.8 phosphate buffer) and drug release from the formulation was uniform (Figure 4). The results showed that the optimized dissolution media could be employed to conduct dissolution testing of NEM tablets.

CONCLUSION

It was concluded that the two stable UV-spectrophotometric methods were developed and successfully applied as dissolution media to test *in-vitro* bioequivalence studies of nelfinavir mesylate. Analytical methods using optimized media were developed and they can be used for routine assay

of NEM in various dosage forms. The developed dissolution media could be employed as simulated gastric fluid (0.1 M HCl) and simulated intestinal fluid (pH 7.8 phosphate buffer) to study the *in-vitro* dissolution profiles of tablets of NEM. Further these methods could be extended to bioequivalence studies of newly developed formulations of NEM in the selected liquid media for both conventional and extended release dosage forms.

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