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Original Article

DEVELOPMENT OF SPECTROPHOTOMETRIC AND FLUOROMETRIC METHODS FOR ESTIMATION OF DARUNAVIR USING QBD APPROACH

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ABSTRACT

Objective: The main objective of the present study is to develop newer simple, precise spectrophotometric and fluorometric methods of estimation for Darunavir using coupling agent *O*-pthaladehyde.

Methods: The experimental work was designed for both spectroscopic and fluorometric method development and validation. The method is based on formation complex of Darunavir with *O*-pthaladehyde. QbD approach was applied by varying different parameters. These parameters were designed into Ishikawa diagram.

Results: The complex Darunavir-Phthalaldehyde in methanol with 0.1 N HCl showed linearity for both spectrophotometric and fluorometric methods. The calibration curve by spectrophotometry is linear in concentration range of 2-22 µg/ml with regression coefficient (R^2) = 0.998 at 355 nm and for fluorometry it is linear in concentration range of 0.5-5.0 ng/ml with regression coefficient (R2) = 0.999. This method was found to be rugged and robust in different testing criteria with % RSD less than 2. The limit of detection and limit of quantification was found to be 0.2 µg/ml and 0.8 µg/ml for a spectrophotometric method and 0.12 µg/ml and 0.43 µg/ml for fluorometric method respectively.

Conclusion: Both methods were found to be precise with % RSD of less than 2. The % recovery of the spectrophotometric and fluorometric methods was found to be 101.04 %, 98.15 % respectively. In this way, the results of all validation parameter were within the limits as per International Conference on Harmonization guideline.

Keywords: Spectrophotometry, Fluorometry, Darunavir, Condensation reaction

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INTRODUCTION

Darunavir (DRV) is an anti-retroviral drug and is inhibitor of the human immunodeficiency virus (HIV) protease in adults and children of age 6 y and older [1]. It is a second-generation protease inhibitor, which is discovered to overcome the problems with early protease inhibitor (PIs) like severe side effects and drug toxicities, require a high therapeutic dose, are costly to manufacture, and show a disturbing susceptibility to drug-resistant mutations. DRV is used with ritonavir and other medications to treat HIV [2]. It works by slowing the spread of HIV in the body. Chemically it is [(1*S*, 2R)-3-[(4-aminophenyl) sulfonyl] (2-methylpropyl) amino]-2-hydroxy-1-(phenylmethyl) propyl]-carbamic acid (3*R*, 3a*S*, 6a*R*) hexahydrofuro [2, 3-b] furan-3-yl ester monoethanolate [3, 4] (fig. 1).

The Quality by Design (QbD) principles have been used to advance product and process quality, particularly in automotive industry, is systematic, risk-based approach in pharmaceutical product and analytical method development [5-7].

The three ICH guidelines which throw light upon quality-by-design and related aspects include Q8 Pharmaceutical development, Q9 Pharmaceutical risk management and Q10 Pharmaceutical Quality systems. In fact, the ICH guideline Q8 is sub-divided into two parts: part one deal with pharmaceutical development and Part II is the annex to the guideline which states the principles for Quality-by-Design (QbD) [8-10].

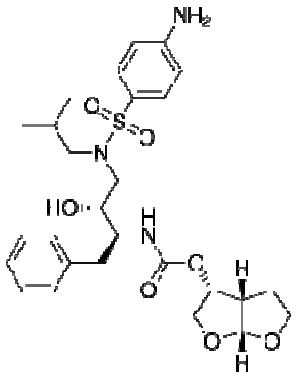

Fig. 1: Structure of darunavir ethanolate

There are various analytical methods developed for Darunavir [11] and drugs which can be made measurable by converting the drug into the complex by coupling reaction [12-14]. In the present study we have developed simple, precise, accurate, two analytical methods to determine the Darunavir, coupling it with *o*-Phthalaldehyde in methanolic media by a condensation reaction (fig. 2).

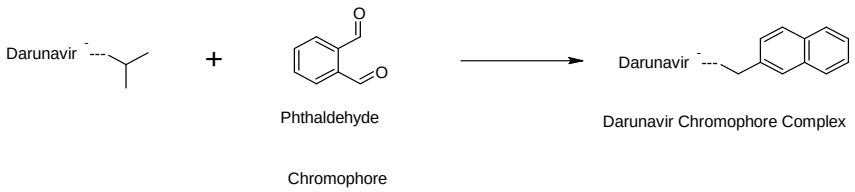

Fig. 2: Formation of darunavir-*O*-phthalaldehyde complex

Table 1: List of materials used for research work

Materials	Source
Darunavir	Cipla Pvt. Ltd
O-Phthalaldehyde	Sigma Aldrich
0.1N Sodium Hydroxide	Laysan Bio Inc.
0.1 N Hydrochloric acid	Fine Chemical Industries, Mumbai
Methanol	Fine Chemical, Mumbai
Distilled water	-

Table 2: List of instruments/Equipments used for research

Instruments/Equipments	Source
Electronic Balance	Shimadzu AUW 220D
Magnetic Stirrer	Remi Equipment Pvt. Ltd.
Centrifuge	Remi Equipment Pvt. Ltd.
UV-Visible Spectrophotometer	Agilent Technology Carry 60 UV-vis UV-1800 Shimadzu, Japan
Micropipettes	Swastik Instrument Private Ltd. Mumbai
Ultrasonicator	Quality Equipment and Instrument
Mechanical Shaker	Remi Equipment Pvt. Ltd
Fluorometer	Systronics

MATERIALS AND METHODS

The material mentioned (table 1) in the procedure are all pure and analytical grade and equipment and machinery used in the procedure are prior to use are calibrated (table 2).

Method development by QbD approach

Method development using Quality by Design approach can be divided into following steps:

- Definition of method goals
- Risk assessment
- Design of experiment with optimization
- MODR (Method Operable Design Region), working point selection and verification
- Method Control Strategy based on the knowledge gained about the developed method [15-20].

Definition of method goals

The primary aim was to develop a more robust method and validation of the developed method. Quality by Design approach was applied to get MODR (Method Operable Design Region). MODR is defined as the set of method parameters over which the robustness and ruggedness experimentation has shown the method can meet the requirements of the ATP [21-23].

Risk assessment

In this stage of QbD approaches critical parameters that affect the overall quality of method were identified such as pH, wavelength, scan speed, temperature and stirring time of complex prepared.

Design of experiment

In the design of the experiment, factors like nature of the solvent, pH of the complex and stirring time were considered.

Preparation of complex of darunavir with coupling reagent

The complex of Darunavir with *O*-Phthalaldehyde was prepared in methanol by three different strategies

a. The complex of Darunavir was prepared by a coupling reaction with *O*-Phthalaldehyde in methanol.

b. The complex of Darunavir was prepared by a coupling reaction with *O*-Phthalaldehyde in methanol using 0.1N sodium hydroxide.

c. The complex of Darunavir was prepared by a coupling reaction with *O*-Phthalaldehyde in methanol using 0.1 N Hydrochloric acid.

Preparation of standard stock solutions

Accurately weighed 10 mg Darunavir was dissolved in 10 ml methanol in a volumetric flask (1000 µg/ml). 1 ml sample from (1000 µg/ml) solution was diluted up to 10 ml with methanol so as to get the solution of concentration (100 µg/ml). Then 1 ml sample from (100 µg/ml) solution was diluted up to 10 ml with methanol so as to get the solution of concentration (10 µg/ml). This solution was treated as stock solutions and three sets of dilutions were prepared by using these stock solutions.

Preparation of working solutions and determination of λ max

Three different types of working solutions of varying concentration were prepared by using standard stock solutions, 0.1 % w/v *O*-Phthalaldehyde solution in methanol, 0.1 N NaOH solutions and 0.1 N HCl solutions.

Preparation of working solution by using standard stock solutions and 0.1 % w/v *O*-Phthalaldehyde solution

0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2 ml of solutions were removed from standard stock solution of concentration 100 µg/ml to these 1.0 ml of 0.1 % w/v *O*-Phthalaldehyde solution was added and final volume was adjusted up to 10 ml with methanol so as to obtain final concentrations of 2,4,6µg/ml etc.

Spectrophotometric method: The λmax was determined by scanning one of the solutions over the range of 200-800 nm by using UV-Visible spectrophotometry and λmax was found to be 249 nm in methanol.

Flourometric method: The dilute solutions of conc.0.5-5.0 ng/ml were prepared and analysed by fluorometry.

Preparation of working solution by using standard stock solutions, 0.1 % w/v *O*-Phthalaldehyde solution and 0.1 N Hydrochloric acid

0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2 ml of solutions were removed from standard stock solution of concentration 100 µg/ml to these 1.0 ml of 0.1 % w/v O-Phthalaldehyde solution and 1 ml 0.1 N Hydrochloric acid were added to each and final volume was adjusted upto 10 ml with methanol so as to obtain final concentrations of 2.0, 4.0, 6.0 µg/ml etc. Spectrophotometric method: The λmax was determined by scanning one of the solutions over the range of 200-800 nm by using UV-Visible spectrophotometry and λmax was found to be 355 nm in 0.1N Hydrochloric acid.

Flourometric method: The dilute solutions of conc.0.5-5.0 ng/ml were prepared and analysed by fluorometry.

Preparation of working solution by using standard stock solutions, 0.1 % w/v *O*-Phthalaldehyde solution and 0.1 N Sodium hydroxide

0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2 ml of solutions were removed from standard stock solution of concentration 100 µg/ml

to these 1.0 ml of 0.1 % w/v *O*-Phthalaldehyde solution and 1 ml 0.1 N sodium hydroxide solution were added to each and final volume was adjusted upto 10 ml with methanol so as to obtain final concentrations of 2.0, 4.0, 6.0 µg/ml etc.

Spectrophotometric method: The λ_{max} was determined by scanning one of the solutions over the range of 200-800 nm by using UV-Visible spectrophotometry and λ_{max} was found to be 367 nm in 0.1N Sodium hydroxide.

Flourometric method: The dilute solutions of conc.0.5-5.0 ng/ml were prepared and analysed by fluorometry.

Construction of calibration curve

The absorbance of dilutions prepared above was measured at respective λ max found for each complex against methanol as a blank and recorded. The calibration curve was plotted using absorbance v/s concentration. Then the linear equation and regression coefficient were calculated.

Validation of the analytical method developed

After development and optimization of the method, the following parameters were evaluated: Selectivity, robustness, linearity, limit of detection, limit of quantification, precision and accuracy. These parameters were evaluated according to the standards set by ICH. A risk-based approach based on the QbD principles set out in ICH Q8 and Q9 was applied to the evaluation of method robustness and ruggedness. Structured methodologies for risk assessment, such as Fishbone diagram can be implemented to identify the potential risk of the method due to a small change in method parameters or under a variety of conditions such as different laboratories, analysts, instruments, reagents, days, etc.

Linearity and range

The linearity of an analytical method is its ability to produce test results that are directly proportional to the concentration of an analyte in the sample within a given range. Linearity and range of optimized method were determined by taking absorbance of dilutions prepared of DRV-PTH complex in methanol with 0.1 N HCl at 355 nm using methanol as a blank and plotting the graph of absorbance v/s concentration. Finally, the linear equation and regression coefficient was calculated and the range was decided.

Accuracy and recovery study

This study was carried out using the 20 tablets (Prezista). 20 tablets were weighed and powdered. The powder sample equivalent to 300 mg of active ingredients was weighed and dissolved in 300 ml of methanol (1000µg/ml) and allowed to sonicate for 10 min. The study was performed at three levels by preparing sample solution concentration of 2.0µg/ml, 4.0µg/ml and 6.0µg/ml using a solution of concentration 10µg/ml. The readings (absorbance) of these concentrations were recorded. Then the % RSD of the concentrations was calculated.

The accuracy of the proposed methods was assessed by recovery studies at three different levels. Recovery studies were carried out by standard addition method. It was performed by adding a known amount of DRV solution of the drug to pre-analyzed tablet solutions. The resulting solutions were then reanalyzed by proposed methods.

Precision

The precision is a measure of the degree of reproducibility or repeatability of an analytical method. It provides an indication of random error. The precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variance of a series of measurements. The two types of precision study intra-day and inter-day were performed by analyzing the diluted working solutions for three times within a day (intra-day) and analyzing the same solutions for three different days (interday) precision study.

LOD and LOQ

The limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ were determined by using following formulae.

$$LOD = \frac{3.3\,\sigma}{S} LOQ = \frac{10\,\sigma}{S}$$

σ = Standard deviation

S= slope of the calibration curve

Ruggedness

The ruggedness of analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different instruments, different lots of reagents, different assay, temperatures, different days, different analysts, etc.

The ruggedness of analytical method was performed by analyzing the diluted working solutions by using Agilent and Shimadzu UV-Visible spectrophotometer and by two different analysts.

RESULTS AND DISCUSSION

Determination of λ max

The λ_{max} of complex solutions and plane DRV in methanol, Sodium hydroxide and Hydrochloric acid observed are reported (table 3) and represented (fig. 3 to 6).

Table 3: Wavelength of maximum absorbance (λ_{max})

Complex	λ max (nm)
DRV in methanol	255
DRV-PTH complex in Methanol	249
DRV-PTH complex in Methanol with 0.1 N NaOH	367
DRV-PTH complex in Methanol with 0.1 N HCl	355

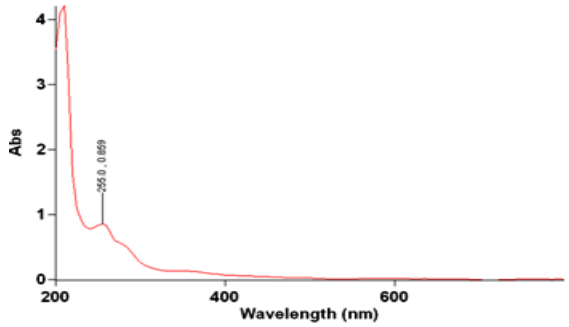

Fig. 3: UV-Visible absorption spectrum of DRV in methanol

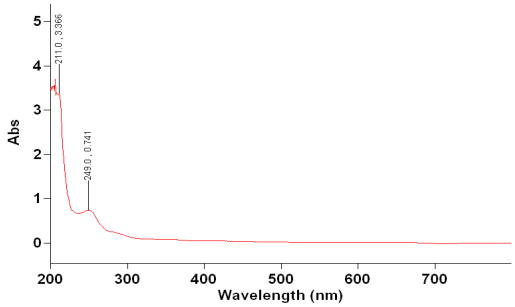

Fig. 4: UV-Visible absorption spectrum of DRV-PTH complex in methanol

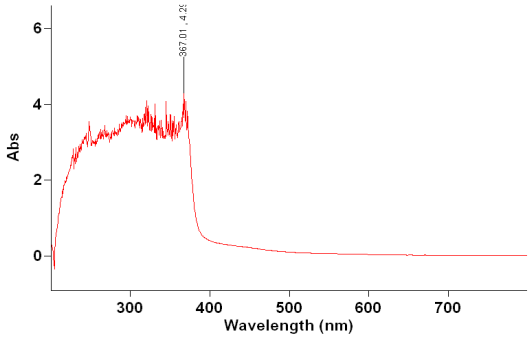

Fig. 5: UV-Visible absorption spectrum of DRV-PTH complex in methanol with 0.1 N NaOH

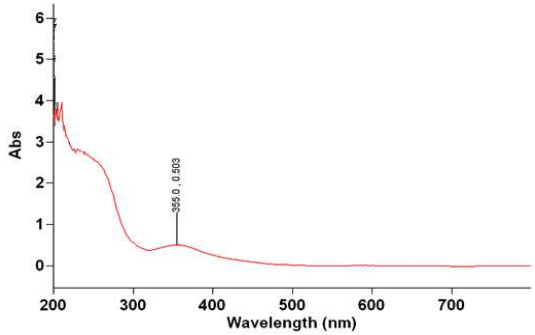

Fig. 6: UV-Visible absorption spectrum of DRV-PTH complex in methanol with 0.1 N HCl

Linearity curve of complexes

The graph of concentration vs. absorbance of three complexes was plotted. It was found that the graph of DRV-PTH complex in Methanol and DRV-PTH complex in Methanol with 0.1 N NaOH was not linear while that of DRV-PTH complex in Methanol with 0.1 N HCl was linear in concentration range 2.0-22 µg/ml at 355 nm. The DRV-PTH complex in Methanol with 0.1 N HCl obeys Beer-Lamberts law in the range 4.0-22.0 µg/ml by spectrophotometric method. The linearity curve of DRV-PTH complex in methanol with 0.1 N HCl using spectrophotometric method was shown (fig. 7). Fluorometric method for DRV-PTH complex in Methanol and DRV-PTH complex in Methanol with 0.1 N NaOH was not linear while that of DRV-PTH complex in Methanol with 0.1 N HCl was linear in concentration range 0.5-5.0 ng/ml at 355 nm. The linearity curve of DRV-PTH complex in methanol with 0.1 N HCl by fluorometric method was shown in (fig. 8).

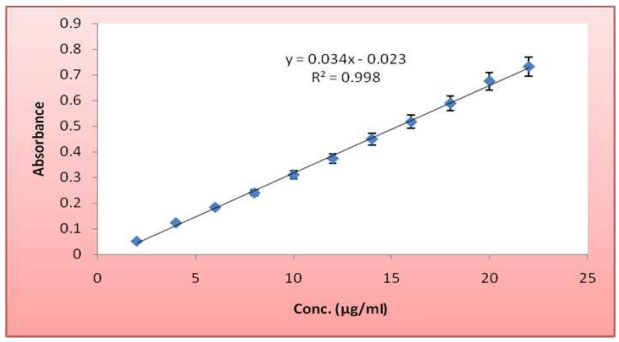

Fig. 7: Linearity curve of DRV-PTH complex in methanol with 0.1 N HCl by agilent UV-visible spectrophotometer

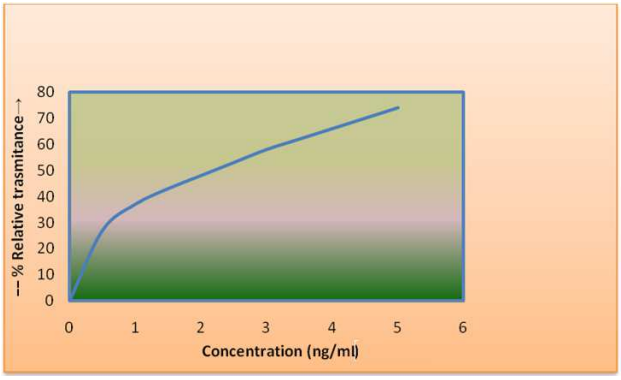

Fig. 8: Linearity curve of DRV-PTH complex in methanol with 0.1 N HCl by a fluorometer

Table 4: Linearity and range parameters for spectrophotometric method

Statistical parameters	Values
Correlation coefficient	0.998
Slope	*0.034*
Intercept	*0.023*
Range	*2-22 µg/ml*
Standard deviation	0.0035
RSD	*0.0077*

Table 5: Linearity and range parameters for fluorometric method

Statistical parameters	Value
Correlation	0.997
Slope	17.72
Intercept	2.954
Range	0.5-5ng/ml
SDV	0.763
RSD	0.01

The critical parameter extracted for both methods were studied (table 6 and 7). The Ishikawa fishbone diagram for spectrophotometric and fluorometric methods was also studied (fig. 9 and 10).

Table 6: Critical attributes extracted for spectrophotometric method

Parameters	Extracted result
pH	Acidic
Wavelength	355 nm
Scan speed	Medium
Temperature	0-8 ° C and 30-35 °C
Stirring time	5 and 10 min

Table 7: Critical attributes extracted for fluorometric method

Parameters	Extracted result
pH	Acidic
Sensitivity	0.2 and 2
Temperature	0-8 ° C and 30-35 °C
Stirring time	5 and 10 min

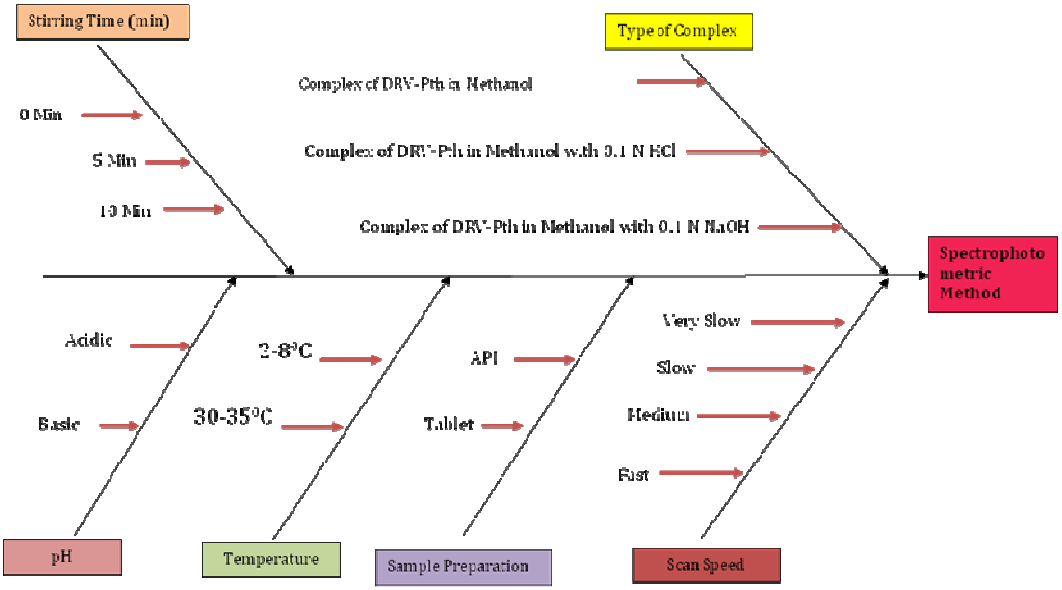

Fig. 9: Ishikawa diagram for a spectrophotometric method

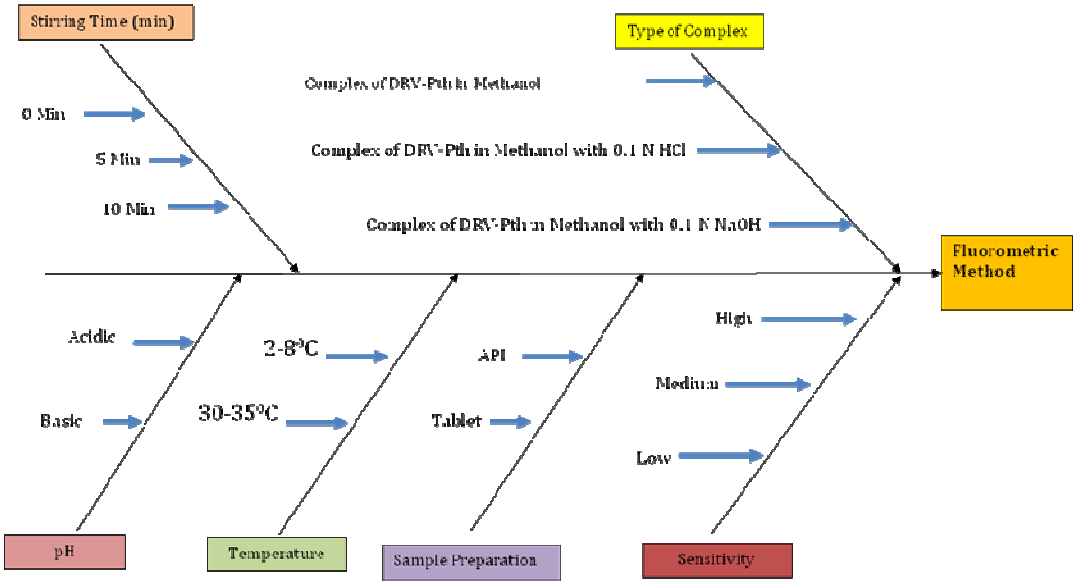

Fig. 10: Ishikawa diagram for a fluorometric method

Validation of analytical method

The above-optimized method of DRV-PTH complex in methanol with 0.1 N HCl was validated for a different parameter like precision, accuracy and recovery, LOD, LOQ, robustness and ruggedness for both analytical methods (table 8 and 9). The values of analytical parameters were found to be within standard limits.

Precision (Repeatability)

The spectrophotometric method for DRV-PTH complex in methanol with 0.1 N HCl was found to be precise as indicated by intraday and interday analysis showing % RSD less than 2. The intraday and interday precision values observed were calculated. The fluorometric method for DRV-PTH complex in methanol with 0.1 N HCl was also found to be precise as indicated by intraday and interday analysis showing % RSD less than 2. The intraday and interday precision values observed by a fluorometric method.

Accuracy and recovery

The accuracy of the method was performed by calculating % recovery of Darunavir by the standard addition method**.** The % recovery of Darunavir by spectrophotometric method was found to be 101.04 % and 98.15 % by a fluorometric method. Accuracy data by spectrophotometric and fluorometric method (fig. 11).

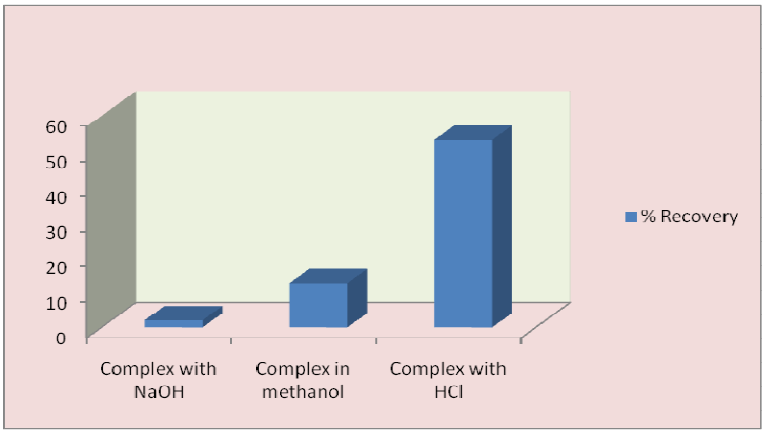

Fig. 11: Accuracy data by the spectrophotometric method

Limit of detection

The limit of detection by spectrophotometric and fluorometric method was found to be 0.2 µg and 0.129 ng respectively.

Limit of quantification

The limit of quantification by spectrophotometric and fluorometric method was found to be 0.8 µg and 0.43 ng respectively.

Robustness

The robustness of analytical method at various temperatures and stirring time was performed and the results of the same are presented for a spectrophotometric method and fluorometric method. There was no significant difference observed in % RSD at 2-8 °C and 30-35 °C and different stirring time by the spectrophotometric and fluorometric method.

Table 8: Statistical data of validation by spectrophotometric method

Statistical parameter	Value
λ max	355 nm
Correlation Coefficient	0.998
Slope	0.034
Intercept	0.023
Range	*2-22 µg/ml*
SD	0.0035
RSD	0.0077
Precision (% RSD)	
Intraday	0.440
Interday	0.459
Recovery	*101.04%*
LOD	0.2
LOQ	0.8

Table 9: Statistical data of validation by fluorometric method

Statistical parameter	Value
Correlation Coefficient	0.997
Slope	17.72
Intercept	2.954
Range	*0.5-5 ng/ml*
SD	0.763
RSD	0.01
Precision (% RSD)	
Intraday	1.774
Interday	0.201
Recovery	*98.15 %*
LOD	0.129
LOQ	0.43

Ruggedness

The ruggedness study was performed by two different analysts in the same laboratory. There was reproducibility in the result obtained by two different analysts.

CONCLUSION

Two simple, easily accessible and economic analytical methods like spectrophotometric and fluorometric methods were developed by QbD approach for Darunavir. The Darunavir was coupled with O-paraldehyde to form complex that was measured in the UV-Visible spectroscopic region (λmax 355 nm). The same complex was fluorescent and analyzed by Fluorometry thus another analytical method was developed. While developing Spectrophotometric method quality was ensured by changing the experimental environments. Some of the experimental parameters changed to inculcate quality were the pH and physical parameters. The Darunavir and O-Phthalaldehyde complex was first measured in methanol solvent. Then reaction media was made acidic with HCl and response was recorded. With NaOH making media basic the measurements were made and the response was recorded. The methods thus developed were validated for linearity, accuracy specificity, precision, percentage recovery, ruggedness and robustness. The validation parameters were calculated and expressed in statistical terms. It was found that all the values of the parameters were within the normal range. The developed methods were compared and it was concluded that the method in acidic media was more accurate, specific and precise. The interference of fluorescent property of complex was not considered for the UV-Visible Spectroscopic method.

Similarly, the Fluorometric method was also developed with Darunavir complex which was sensitive; easily accessible was validated for linearity, accuracy specificity, precision, percentage recovery, ruggedness and robustness. On the calculation of validation parameters and statistical expression, all the parameters were showed the values within the normal range.

32 factorial design was employed wherein concentration, temperature and scan speed were selected as dependent variables. The factors were operated at 3 levels.

(-1, 0,+1) and linearity was selected as dependent factor.

Thus developed methods are simple, easily accessible, and economic. Does not require stringent expertise in performing and further are of immense useful in quality control and estimation of Darunavir.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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