

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

Talluri Sarada Mrinalini¹, Achanti Suneetha², Marella Vijaya Lakshmi³, Shaik Asha Begum⁴, Ravulapati Anusha⁵, Rajana Manimala⁶, Suragani Pranav Sai⁶, K.V.V.L. Ram Kumar⁶

¹Assistant Professor, Department of Pharmaceutical Analysis, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010.

²Principal, Department of Pharmaceutical Analysis, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010.

³Associate Professor, Department of Pharmaceutical Analysis, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010

⁴Head of Department, Department of Regulatory Affairs, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010.

⁵Assistant Professor, Department of Pharmaceutics, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010.

⁶ B. Pharmacy, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, INDIA, 520010.

Corresponding Author: T. Sarada Mrinalini

Email Id: Mrinalini.talluri@gmail.com

Received: 16th August, 2026; Revised: 8th September 2026; Accepted: 19th September, 2026; Available Online: 20th September, 2026

Abstract

The simultaneous measurement of doxecitine and doxribtimine in pharmaceutical dosage forms and bulk medicinal substances, a stability-indicating reversed-phase high-performance liquid chromatographic technique was created and verified. Using an isocratic mobile phase made up of acetonitrile and 0.1% formic acid in a 20:80, v/v ratio, chromatographic separation was accomplished on a Waters X-Bridge Phenyl column (250 × 4.6 mm, 5 µm). A photodiode array detector was used for detection at 229 nm after the mobile phase was supplied at a flow rate of 1.0 mL/min. With a resolution of 5.88, doxecitine and doxribtimine were eluted with retention periods of 4.582 and 3.204 minutes, respectively. The corresponding theoretical plate counts were 5,222 and 4,091, while the tailing factors were 1.02 and 1.23.

For both analytes, the technique was linear over the concentration range of 25–150 µg/mL, with correlation values of 0.99982 for doxecitine and 0.99983 for doxribtimine. %RSD values of 0.77% and 0.21% were displayed by system precision, whereas method precision values were 0.69% and 0.76%, respectively. Mean recoveries were 99.8% for doxribtimine and 99.7% for doxecitine. The assay results were 98.8% and 100.2%, respectively. For both analytes, the limits of detection and quantitation were 0.60 and 2.00 µg/mL, respectively. There was no evidence of interference from blank or placebo components. With %RSD values not going above 1.00%, the approach held up well after intentional changes in flow rate and mobile-phase composition.

Forced-degradation studies demonstrated degradation of 0.8–13.1% for doxribtimine and 1.6–14.1% for doxecitine, with maximum degradation observed under oxidative conditions. In all stress conditions, the peak-purity angles remained below their respective purity thresholds, confirming the absence of co-eluting degradation products. The developed method was therefore specific, accurate, precise, sensitive, robust, and suitable for routine quality-control and stability analysis of doxribtimine and doxecitine in bulk and pharmaceutical formulations.

Keywords: Doxribtimine; doxecitine; RP-HPLC; simultaneous determination; method validation; forced degradation; stability-indicating method.

How to cite this article: Talluri Sarada Mrinalini¹ Achanti Suneetha² Marella Vijaya Lakshmi³ Shaik Asha Begum⁴ Ravulapati Anusha⁵ Rajana Manimala⁶ Suragani Pranav Sai⁶ K.V.V.L. Ram Kumar⁶. An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions. *Int J Drug Deliv Technol.* 2026;16(84s):1961-1974. DOI: 10.25258/ijddt.16.84s.198.

1. Introduction

Hymidine Kinase 2 deficiency (TK2d) is a rare, autosomal recessive mitochondrial DNA maintenance condition caused by pathogenic mutations in the nuclear TK2 gene. The mitochondrial thymidine kinase 2 enzyme catalyses an essential step in the phosphorylation of pyrimidine deoxynucleosides required for maintaining mitochondrial deoxyribonucleotide pools. Deficiency of this enzyme results in mitochondrial DNA depletion, multiple mitochondrial DNA deletions, or both, and predominantly manifests as a progressive mitochondrial myopathy. The clinical manifestations may include

progressive skeletal-muscle weakness, loss of motor function, respiratory insufficiency, and premature mortality.[1,2]

Pyrimidine nucleosides called doxecitine and doxribtimine are used in conjunction to treat TK2d. The chemical formula for doxecitine is 4-amino-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-one. Its molecular weight is 227.22 g/mol. The chemical formula for doxribtimine is 1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1H,3H)-dione. Its molecular

weight is 242.23 g/mol. The combination, which is a 1:1 blend of doxecitine and doxribtimine, has been approved for the treatment of TK2d in both adult and pediatric patients whose symptoms started at or before the age of twelve.[3]

The therapeutic combination functions as a nucleoside substrate-enhancement therapy by increasing the availability of deoxycytidine- and deoxythymidine-derived nucleotide precursors. Following cellular uptake, doxecitine and doxribtimine can be phosphorylated through intact cytosolic salvage pathways involving deoxycytidine kinase and thymidine kinase 1. The resulting nucleotide precursors support the replenishment of mitochondrial deoxyribonucleotide pools and mitochondrial DNA synthesis. Preclinical studies demonstrated that deoxynucleoside supplementation increased mitochondrial DNA copy number and improved disease-related outcomes in TK2-deficient models, while cytosolic salvage-pathway enzymes were identified as important determinants of the therapeutic response.[3–5]

Reliable analytical procedures are required to establish the distinctiveness, strength, purity, and stability of drug substances and finished pharmaceutical products. Simultaneous determination of active components in a combined formulation requires adequate selectivity, resolution, accuracy, precision, sensitivity, and robustness. RP-HPLC is particularly suitable for pharmaceutical assay and stability testing because it permits efficient separation and quantitative determination of multiple analytes under controlled chromatographic conditions. Analytical procedure development and validation should prove that proposed method is suitable for its proposed purpose and provides reliable results throughout the defined analytical range.[6,7]

Published investigations on doxecitine and doxribtimine have mainly addressed their pharmacokinetics, safety, therapeutic application, and analysis in biological matrices. In a phase 1 investigation, plasma and urine concentrations of both nucleosides were determined using validated liquid chromatography–mass spectrometric procedures to characterize their pharmacokinetic profiles and food effects.[8] To the best of our knowledge, however, no validated stability-indicating RP-HPLC method has been described for the simultaneous measurement of doxecitine and doxribtimine in pharmaceutical dosage forms and bulk medicinal substances. The lack of such a process demonstrated the requirement for a readily available analytical technique for standard assay and stability assessment.

In order to determine doxecitine and doxribtimine simultaneously in bulk drug substances and pharmaceutical dosage forms, the current work was conducted to design and optimize a quick, affordable, and stability-indicating RP-HPLC method. In compliance with ICH Q2(R2) guidelines, the method was validated for specificity, linearity and range, accuracy, precision, detection limit, quantification limit, and robustness. To determine the specificity and stability-indicating capabilities of the suggested approach, forced-degradation investigations were also

carried out under acidic, alkaline, oxidative, reductive, thermal, photolytic, and hydrolytic conditions.[7]

2. Materials And Methods

2.1 Instrumentation and Apparatus

Chromatographic study was achieved using a Waters Alliance e2695 HPLC system armed with an isocratic pump and Empower 2.0 software for data acquisition and processing. A Eutech pH 700 pH meter was used for pH measurements, while a Sartorius BSA224S-CW analytical balance was used for accurately weighing the standards and chemicals. An Unichrome UCA 701 ultrasonicator was employed for the dissolution and degassing of solutions. Class-A pipettes, burettes, beakers, and other volumetric glassware manufactured by Borosil were used throughout the analytical procedure.

2.2. Reagents and Chemicals

For the study, we purchased formic acid of analytical reagent grade and acetonitrile of HPLC grade from Merck. An internal Milli-Q water purification system provided HPLC-grade water. During the technique development and validation process, all solvents and reagents were of appropriate analytical or HPLC quality and were utilized without additional purification.

2.3 Selection of Detection Wavelength

The absorption spectra of doxribtimine and doxecitine were recorded using a photodiode array detector over the wavelength range of 200–400 nm. The solutions were prepared in a diluent consisting of acetonitrile and 0.1% formic acid in the ratio of 20:80, v/v, and the same diluent was used as the blank. Both analytes exhibited adequate absorbance and detector response at 229 nm. Therefore, 229 nm was selected as the common detection wavelength for the simultaneous RP-HPLC determination of doxribtimine and doxecitine.

2.4 Optimization of Chromatographic Conditions

Various chromatographic conditions were evaluated during method development by altering parameters such as the stationary phase, mobile-phase composition, flow rate, and detection wavelength. Several experimental trials were performed to obtain acceptable retention, peak symmetry, theoretical plate count, and resolution between the analytes. The chromatographical conditions that produced well-resolved and symmetrical peaks within a suitable run time were selected as the optimized conditions for further method validation.

3. Preparation Of Solutions

3.1 Standard Stock Solution

A 10 mL volumetric flask was filled with accurately weighed amounts of doxribtimine and doxecitine working standards, 10 mg each. A appropriate amount of diluent was added, and the mixture was sonicated until the medicines were thoroughly dissolved. To create standard stock solutions with 1,000 µg/mL of doxribtimine and doxecitine, the volume was increased with the same diluent until the mark was reached.

3.2 Standard Working Solution

A milliliter of the standard stock solution was transferred to a 10 mL volumetric flask and diluted to the mark with the diluent. The final working standard solution contains 100 µg/mL of doxribtimine and doxecitine.

3.3 Sample Solution

A 10 mL volumetric flask was filled with an accurately weighed quantity of the pharmaceutical formulation containing 10 mg each of doxribtimine and doxecitine. A appropriate amount of diluent was added, and the solution was sonicated for 30 minutes to guarantee full extraction of the analytes. The volume was then increased to the desired level using the same diluent and well mixed. A milliliter of the resulting sample stock solution was transferred to a 10 mL volumetric flask and diluted to the mark with the diluent. The final sample solution had 100 µg/mL of doxribtimine and doxecitine. The fluid was filtered using a 0.45 µm membrane filter before injection.

4. Method Development

During technique development, six chromatographic trials were carried out with different stationary and mobile phase compositions. The injection volume and flow rate were set at 10 µL and 1.0 mL/min, respectively. Table 1 summarizes the conditions and observations from each trial.

Table 1: Chromatographic conditions evaluated during method optimization

Trial	Column	Mobile phase composition, v/v	Detection condition	Flow rate	Injection volume	Run time	Observation
Trial 1	Luna C18 (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% TFA (80:20)	PDAScans: 200–400 nm	1.0 mL/min	10 µL	6 min	Doxribtimine eluted too early; therefore, the retention behaviour was considered

							unsuitable.
Trial 2	Luna C18 column with dimensions (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% TFA (70:30)	229 nm	1.0 mL/min	10 µL	3.5 min	Theoretical plate counts were inadequate, and the tailing factor of doxecitine exceeded the acceptable limit.
Trial 3	Luna C18 (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% formic acid (50:50)	229 nm	1.0 mL/min	10 µL	Broad peaks were observed;	therefore, the conditions were not selected.
Trial 4	Waters	Acetonitrile	229 nm	1.0 mL/min	10 µL	Peak splitting	the resolution

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

al 4	X- Brid ge Phe nyl (25 0 × 4.6 mm , 5 µm)	le– form ic acid (40:6 0)		L/ mi n		ing and inade quate separ ation were obser ved;	ution was only 1.93.									mete rs were obtai ned. Thes e cond ition s were selec ted for meth od valid ation .
T ri al 5	Wat ers X- Brid ge Phe nyl (25 0 × 4.6 mm , 5 µm)	Acet onitri le– 0.1% form ic acid (30:7 0)	229 nm	1.0 m L/ mi n	10 µL	The basel ine respo nse was unsat isfact ory										
T ri al 6	Wat ers X- Brid ge Phe nyl (25 0 × 4.6 mm , 5 µm)	Acet onitri le– 0.1% form ic acid (20:8 0)	229 nm	1.0 m L/ mi n	10 µL	7 min	Well - resol ved, sym metri cal peak s with satisf actor y syste m- suita bility para									

4.1 Selection of the Optimized Chromatographic Conditions

Among the six trials, Trial 6 produced satisfactory chromatographic separation and system-suitability results. The analysis was performed Using a Waters X-Bridge Phenyl column (250 × 4.6 mm, 5 µm) with an isocratic mobile phase consisting of acetonitrile and 0.1% formic acid in a 20:80 v/v ratio. The mobile phase was given at a flow rate of 1.0 mL/min, with an injection volume of 10 µL and detection at 229 nm. The overall chromatographic run time was 7 minutes. Under the optimized conditions, doxribtimine was eluted at 3.204 min with a peak area of 1,379,230, a theoretical plate count of 5,222, and a tailing factor of 1.02. Doxecitine was eluted at 4.582 minutes with a peak area of 2,945,167, a theoretical plate count of 4,091, and a tailing factor of 1.23. The resolution between the two analyte peaks was 5.88. Therefore, Trial 6 was selected as the optimized chromatographic method and was subsequently subjected to analytical method validation.

4.2 Mobile Phase

The mobile phase was produced by combining acetonitrile and 0.1% formic acid in a 20:80 v/v ratio. To ensure accurate chromatographic analysis, the mobile phase was well mixed and filtered through a 0.45 µm membrane filter. It was then degassed before use.

4.3 Optimized Chromatographic Conditions

Chromatographic analysis was accomplished using a suitable high-performance liquid chromatographic system armed with an autosampler and a PDA detector. Separation was achieved under isocratic conditions using the optimized chromatographic parameters given below in table 2.

Table 2: Optimized Chromatographic Conditions

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

Parameter	Condition
HPLC system	HPLC system armed with an autosampler and PDA detector
Column Details	Waters X-Bridge Phenyl (250 × 4.6 mm, 5 µm)
Mobile phase composition	Acetonitrile–0.1% formic acid (20:80, v/v)
Mode of elution	Isocratic
Detection wavelength	229 nm
Mobile phase Flow rate	1.0 mL/min
Injection volume	10 µL
Sample Run time	7 minutes
Column temperature	Ambient temperature, approximately 25°C

4.4 Chromatographic Procedure

The chromatographic apparatus was equilibrated with the mobile phase until a consistent baseline was established. The HPLC system received separate injections of 10 µL for the standard and sample solutions. Chromatograms were recorded at 229 nm using a photodiode array detector. The peaks corresponding to doxribtimine and doxecitine were identified based on their respective retention times, and their peak areas were noted. The purity of each analyte was calculated by comparing the sample peak area with the corresponding standard peak area after considering the standard purity, dilution factors, sample quantity, and label claim.

5. Method Validation

5.1 System-Suitability Testing

System suitability was evaluated using six replicate injections of the standard solution containing doxribtimine and doxecitine. Retention time, peak area, theoretical plates, tailing factor, resolution, and %RSD were determined. Acceptance criteria were $\geq 2,000$ theoretical plates, tailing factor ≤ 2.0 , resolution ≥ 2.0 , and peak-area %RSD $\leq 2.0\%$.

5.2 Specificity

Specificity was assessed by separately injecting blank, placebo, standard, and sample solutions. Chromatograms were examined for interference at the retention times of doxribtimine and doxecitine, and peak identity and separation were confirmed by comparing standard and sample chromatograms.

5.3 Linearity

A combined standard stock solution of doxribtimine and doxecitine (1,000 µg/mL each) was prepared. Dilutions covering 25–150 µg/mL were analyzed. Calibration

curves of peak area versus concentration were plotted, and linearity was evaluated by least-squares regression analysis.

Table 3: Preparation of Linearity Solutions

Level	Stock aliquot (mL)	Final volume (mL)	Doxribtimine (µg/mL)	Doxecitine (µg/mL)
I	0.25	10	25	25
II	0.50	10	50	50
III	0.75	10	75	75
IV	1.00	10	100	100
V	1.25	10	125	125
VI	1.50	10	150	150

5.4 Accuracy (Recovery Study)

The standard-addition approach was used to measure accuracy at the 80%, 100%, and 120% levels. The diluent was used to create combined standard and sample stock solutions with 1,000 µg/mL doxribtimine and doxecitine concentrations. Table 4 shows how appropriate volumes of the sample and standard stock solutions were transferred into 10 mL volumetric flasks and diluted to volume with the diluent.

Table 4: Preparation of Accuracy Solutions

Accuracy level	Sample stock solution (mL)	Standard stock solution (mL)	Final volume (mL)	Final volume (mL)
80%	1.0	0.8	10	180
100%	1.0	1.0	10	200
120%	1.0	1.2	10	220

Each accuracy solution was prepared in triplicate and injected into the chromatographic system under the optimized conditions. The amount recovered and percentage recovery of doxribtimine and doxecitine were calculated at to each level.

5.5 Precision

Precision was measured in terms of system precision, method precision, and intermediate precision. Six replicate injections of a standard solution with 100 µg/mL of doxribtimine and doxecitine were used to assess system precision. Method precision was determined by analysing six independently prepared sample solutions at the nominal concentration. Intermediate precision was evaluated by repeating the analysis on two different days. Precision was given as the percentage relative standard deviation (%RSD) of the peak areas, with an acceptance criterion of not more than 2.0%.

5.6 Robustness

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

Robustness was assessed by deliberately changing the flow rate and mobile-phase composition. The flow rate was varied between 0.9 and 1.1 mL/min, with the optimal value of 1.0 mL/min. The acetonitrile-0.1% formic acid ratio was changed from 18:82 to 22:78, v/v, with the optimal ratio being 20:80, v/v. A standard solution containing 100 µg/mL of doxribtimine and doxecitine was analyzed for each condition. The impact of these adjustments on retention duration, peak area, resolution, tailing factor, theoretical plate count, and %RSD were investigated. The approach was considered robust when the system-suitability parameters remained satisfactory and the peak-area%RSD was less than 2.0%.

5.7 Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification were determined using the signal-to-noise ratio approach. The concentration producing a signal-to-noise ratio of approximately 3:1 was considered the LOD, whereas the concentration producing a ratio of approximately 10:1 was considered the LOQ. Dilute standard solutions of doxribtimine and doxecitine were injected to determine these sensitivity parameters.

6. Forced-Degradation Studies

To assess the stability-indicating capabilities of the new RP-HPLC technology, forced degradation studies were performed in acidic, alkaline, oxidative, reductive, thermal, photolytic, and hydrolytic conditions. For the solution-state degradation investigations, 20 mg of the combination formulation was employed, which is equivalent to 10 mg of doxribtimine and doxecitine. After stress treatment, the solutions were diluted to the desired concentration, filtered through a 0.45 µm syringe filter, and analyzed using optimal chromatographic conditions.

Table 5: Conditions employed for forced-degradation studies

Stress condition	Degradation procedure
Acid degradation	The material was transferred to a 10-mL volumetric flask, and 1 mL of 1 N HCl was added. The solution was kept at 60°C for 20 minutes, then cooled and neutralized with 1 N NaOH. The volume was increased to the desired level using the diluent. The resultant solution was diluted to 10 mL with the diluent and filtered before analysis.
Alkaline degradation	The sample was transferred to a 10 mL volumetric flask, and 1 mL of 1N NaOH was added. The solution was held at 60°C for 20 minutes, then cooled and neutralized with 1 N HCl. The volume was increased to the

	desired level using the diluent. The resultant solution was diluted to 10 mL with the diluent and filtered before analysis.
Oxidative degradation	The material was transferred to a 10 mL volumetric flask, and 1 mL of 10% hydrogen peroxide was added. The solution was kept at 60°C for 20 minutes and then allowed to cool at room temperature for 15 minutes. The volume was increased with the diluent. Before analysis, one milliliter of the solution was diluted to 10 mL using the diluent and filtered.
Reduction degradation	The sample was transferred to a 10 mL volumetric flask, and 1 mL of 10% sodium bisulfite solution was added. The solution was kept at 60°C for 20 minutes and then cooled to room temperature for 15 minutes. The volume was increased with the diluent. Before analysis, one milliliter of the solution was diluted to 10 mL using the diluent and filtered.
Thermal degradation	The drug sample was placed on a Petri plate and heated in a hot-air oven at 105°C for three hours. After cooling, the exposed sample was dissolved and diluted to the appropriate analytical concentration with the diluent prior to HPLC analysis.
Photolytic degradation	The drug sample was exposed in a photostability chamber for three hours. The exposed sample was then dissolved and diluted to the desired analytical concentration with the diluent prior to HPLC analysis.
Hydrolytic degradation	The sample was transferred to a 10 mL volumetric flask, and 1 mL of HPLC-grade water was added. The solution was kept at 60°C for 20 minutes and

	then cooled to room temperature for 15 minutes. The volume was increased with the diluent. Before analysis, one milliliter of the solution was diluted to 10 mL using the diluent and filtered.
--	---

7. Results And Discussion

7.1 Optimized Liquid chromatographic Conditions

Six chromatographic trials were evaluated by changing the stationary phase and mobile-phase contents to obtain adequate retention, peak symmetry, column efficiency, and resolution of doxribtamine and doxecitine. The mobile phase flow rate and sample injection volume were maintained at 1.0 mL/min and 10 µL, respectively. The outcomes of the optimization trials are summarized in Table 6.

Table 6: Summary of chromatographic method-optimization trials

Trial	Column	Mobile-phase composition (v/v)	Retention time (min) ^a	USP plate count ^a	USP tailing factor ^a	Resolution	Final observation
1	Luna C18 (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% TFA (80:20)	1.408; 3.115	2,153; 2,417	1.25; 1.19	8.60	Doxribtamine eluted at a relatively short retention time; therefore, the conditions were not selected.

2	Luna C18 (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% TFA (70:30)	1.818; 2.919	1,526; 1,042	1.56; 2.08	5.57	Inadequate theoretical plate counts and excessive tailing of the doxecitine peak were observed.
3	Luna C18 (150 × 4.6 mm, 3.5 µm)	Acetonitrile–0.1% formic acid (50:50)	2.019; 3.427	2,532; 2,017	1.32; 1.28	7.03	Broad analyte peaks were observed; hence, the conditions were not selected.
4	Waters X-Bridge Phenyl (25	Acetonitrile–0.1% formic acid (40:60)	2.831; 3.218	2,203; 1,954	1.28; 1.97	1.93	Peak splitting and inadequate resolution

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

	0 × 4.6 mm, 5 µm)						between the analytes were observed.										m-suitability parameters were obtained; therefore, these conditions were selected for validation.
5	Waters X-Bridge Phenyl (250 × 4.6 mm, 5 µm)	Acetonitrile–0.1% formic acid (30:70)	2.913; 4.720	3,625; 2,967	1.11; 1.27	9.01	Although satisfactory separation was achieved, the baseline response was unsuitable.										
6	Waters X-Bridge Phenyl (250 × 4.6 mm, 5 µm)	Acetonitrile–0.1% formic acid (20:80)	3.204; 4.582	5,222; 4,091	1.02; 1.23	5.88	Well-resolved, symmetrical peaks with satisfactory column efficiency and system										

Selection of the Optimized Method

Trial 6 provided satisfactory retention, peak symmetry, column efficiency, and resolution for both analytes. Doxribtimine and doxecitine were eluted at 3.204 and 4.582 min, respectively. Their corresponding theoretical plate counts were 5,222 and 4,091, and the tailing factors were 1.02 and 1.23. A resolution of 5.88 was obtained between the analyte peaks. Therefore, the Waters X-Bridge Phenyl column with acetonitrile and 0.1% formic acid in 20:80%, v/v, was selected as the optimized chromatographic condition for subsequent method validation

Table 7: Optimized Liquid chromatographic system conditions

Instrument Parameter	Conditions
Instrument Used for Analysis	Waters HPLC, auto sampler, PDA detector.
Injection Volume	10µl
Mobile Phase Composition	Acetonitrile: 0.1% Formic acid (20:80)
Column Details	Waters X-Bridge Phenyl column (250mmx4.6mm, 5µm)
Detection Wave Length	229nm

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

Mobile Phase	1 mL/min
Flow Rate	
Chromatogram Runtime	7minutes
Temperature Condition	Ambient temperature (25° C)
Mode of Elution	Isocratic mode of elution

7.2 System suitability: All system suitability parameters were found to be within the specified acceptance limits and were satisfactory in accordance with ICH guidelines.

Table 8: System suitability parameters of Dixribtimine & Doxecitine

S.no	Parameter	Dixribtimine	Doxecitine
1	Retention time	3.204	4.582
2	Plate count	5222	4091
3	Tailing factor	1.02	1.23
4	Resolution	---	5.88
5	%RSD	0.77	0.21

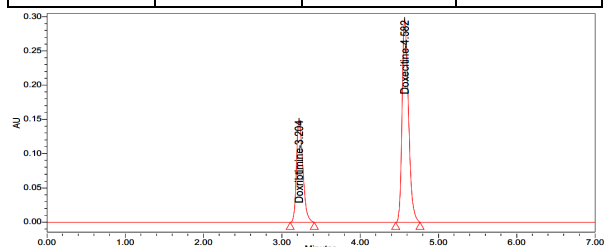


Fig 1: Standard Solution Chromatogram

7.3 Specificity

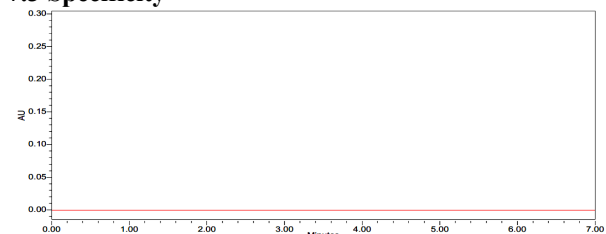


Fig 2: Blank solution Chromatogram

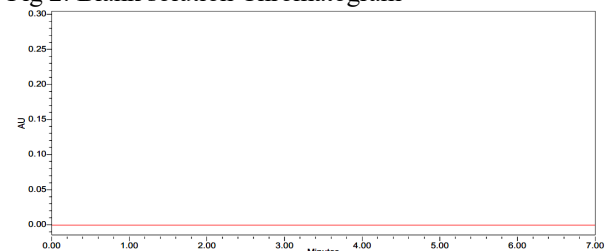


Fig 3: Placebo solution Chromatogram

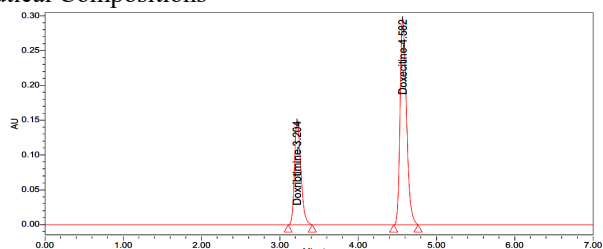


Fig 4: Optimized method chromatogram

Result: No interfering peaks were observed at the retention times of doxribtimine and doxecitine in the blank and placebo chromatograms, demonstrating the specificity and selectivity of the developed chromatographic method.

Table 9. Summary of system-suitability and analytical method-validation results

Parameter	Doxribtimine	Doxecitine	Acceptance criterion
Retention time (min)	3.204	4.582	—
USP theoretical plate count	5,222	4,091	$\geq 2,000$
USP tailing factor	1.02	1.23	≤ 2.0
Resolution between analyte peaks	$\frac{t_{R2} - t_{R1}}{W_{0.05}} = \frac{4.582 - 3.204}{0.588} = 5.88$		≥ 2.0
Linearity range ($\mu\text{g/mL}$)	25–150	25–150	—
Regression equation	$y = 13,518.84x + 8,134.29$	$y = 28,766.95x + 2,588.75$	—

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

Coefficient of determination (R^2)	0.99983	0.99982	≥ 0.999
System precision (%RSD, n = 6)	0.77	0.21	≤ 2.0
Method precision (%RSD, n = 6)	0.69	0.76	≤ 2.0
Intermediate precision (%RSD)	0.86–0.98	0.47–0.77	≤ 2.0
Mean recovery (%)	99.8	99.7	98.0–102.0
Assay (%)	98.8	100.2	98.0–102.0
LOD ($\mu\text{g/mL}$)	0.60	0.60	$S/N \approx 3$
LOQ ($\mu\text{g/mL}$)	2.00	2.00	$S/N \approx 10$

The established technique exhibited satisfactory system suitability, with theoretical plate counts of 5,222 and 4,091 and tailing factors of 1.02 and 1.23 for doxribtimine and doxecitine, respectively. The resolution between the analyte peaks was 5.88. Linearity was demonstrated over the concentration range of 25–150 $\mu\text{g/mL}$, with coefficients of determination of 0.99983 and 0.99982. The %RSD values obtained for system, method, and intermediate precision were below 2.0%. Mean recoveries were 99.8% for doxribtimine and 99.7% for doxecitine, while assay values were 98.8% and 100.2%, respectively. The LOD and LOQ were 0.60 and 2.00 $\mu\text{g/mL}$ for both analytes. These results demonstrated that the method complied with the predefined validation criteria

7.4 Linearity

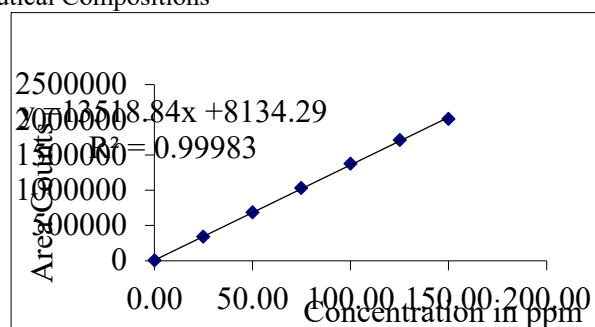


Fig 5: Calibration curve of Dixribtimine

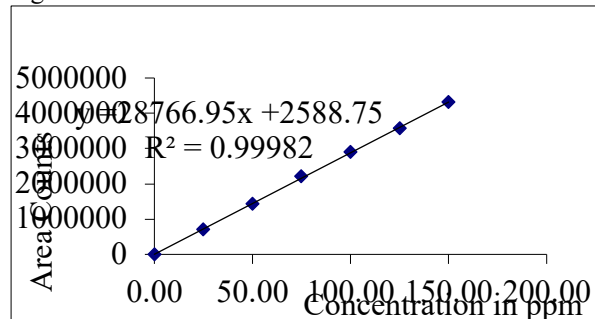


Fig 6: Calibration curve for Doxecitine

Table 10: Linearity Results of Dixribtimine & Doxecitine

Parameter	Dixribtimine	Doxecitine
Regression equation	$y = 13518.84x + 8134.29$	$y = 28766.95x + 2588.75$
Slope	13518.84	28766.95
Intercept	8134.29	2588.75
R^2	0.99983	0.99982

7.5 Accuracy

The standard-addition approach was used to measure accuracy at the 80%, 100%, and 120% levels. Each concentration level was analyzed three times, and the percentage recovery, standard deviation, and percentage relative standard deviation were calculated. The mean recovery rates for doxribtimine ranged from 99.63% to 99.90%, while those for doxecitine ranged from 99.50% to 99.87%. All recovery values were within the acceptable range of 98.0–102.0 percent, with %RSD values less than 2.0 percent, indicating the correctness of the developed approach.

Table 11. Accuracy results of doxribtimine and doxecitine by the RP-HPLC method

Accuracy level	Doxribtimine recovery (%), mean $\pm$ SD (n = 3)	%RSD	Doxecitine recovery (%), mean $\pm$ SD (n = 3)	%RSD

An Optimized Stability-Indicating Rp-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtamine in Pharmaceutical Compositions

80%	99.63 ± 0.55	0.55	99.63 ± 0.21	0.21
100%	99.77 ± 0.45	0.45	99.87 ± 0.15	0.15
120%	99.90 ± 0.40	0.40	99.50 ± 0.30	0.30
Overall mean	99.77 ± 0.42	0.43	99.67 ± 0.25	0.26

Acceptance criterion: Mean recovery of 98.0–102.0% and %RSD not more than 2.0%.

Results interpretation

The overall mean recoveries of doxribtamine and doxecitine were 99.77% and 99.67%, respectively. The low %RSD values indicated satisfactory repeatability of recovery at all three concentration levels. These findings confirmed that the established RP-HPLC method was accurate for the simultaneous quantification of both analytes in the pharmaceutical dosage form.

7.6 Robustness

Robustness was assessed by deliberately changing the flow rate and mobile-phase composition. The flow rate was varied between 0.9 and 1.1 mL/min, with the optimal value of 1.0 mL/min. The amount of acetonitrile in the mobile phase was changed from 18% to 22%, with the optimal composition being acetonitrile-0.1% formic acid (20:80, v/v). The impact of these changes on retention time, tailing factor, theoretical plate count, resolution, and peak-area %RSD were investigated.

Table 12. Robustness results of the developed RP-HPLC method

Parameter varied	Concentration (mL/min)	Doxribtamine Retention Time (min)	Doxribtamine Tailing	Doxribtamine Theoretical Plates	Doxribtamine %RSD	Doxecitine Retention Time (min)	Doxecitine Resolution	Doxecitine Tailing	Doxecitine Theoretical Plates
Flow rate	0.9 mL/min	0.9	3.391	1.06	5.046	1.00	4.753	5.81	1.26

Flow rate	1.0 mL/min	1.0 mL/min	3.204	1.02	5.222	0.77	4.582	5.88	1.23
Flow rate	1.1 mL/min	1.1 mL/min	3.17	0.96	5.568	0.50	4.391	5.44	1.19
Mobile-phase composition	18:82, v/v	18:82, v/v	3.560	1.10	4.703	0.45	4.886	5.68	1.25
Mobile-phase composition	20:80, v/v	20:80, v/v	3.207	1.05	5.271	0.77	4.584	5.84	1.21

An Optimized Stability-Indicating RP-HPLC Process for the Simultaneous Quantification of Doxecitine and Doxribtimine in Pharmaceutical Compositions

M ob ile - ph as e co m po sit io n	2 2: 7 8, v/ v	22: 78, v/v	3.0 21	1.0 1	5,7 12	0. 26	4. 24 5	5. 21	1. 16
---	-------------------------------	-------------------	-----------	----------	-----------	----------	---------------	----------	----------

Deliberate variations in flow rate and mobile-phase composition caused only minor changes in retention times. Tailing factors ranged from 0.96–1.10 for doxribtimine and 1.16–1.26 for doxecitine. Theoretical plate counts remained above 3,500, resolution ranged from 5.21–5.88, and peak-area %RSD was within 1.00% for both analytes. All parameters met the predefined acceptance criteria, confirming the robustness of the developed RP-HPLC method.

7.7 Forced-Degradation Studies

All data fulfilled the acceptance criteria, which included a tailing factor of no more than 2.0, a theoretical plate count of at least 2,000, a resolution of no less than 2.0, and a peak-area%RSD of no more than 2.0%. As a result, the novel RP-HPLC technology was deemed resistant to minor intentional alterations in flow rate and mobile-phase composition.

Table 13. Forced-degradation results for doxribtimine and doxecitine

Str ess co nd itio n	Dox ribt imi ne assa y (%)	Dox ribt imi ne deg rad atio n (%)	P u ri ty a n gl e	Pu rit y th res ho ld	Do xec itin e ass ay (%)	Do xec itin e deg rad atio n (%)	P u ri ty a n gl e	Pu rit y th res ho ld
Co ntr ol	100. 0	0.0	1. 9 5 3	4.1 27	10 0.0	0. 0.0	0. 6 2 3	2.4 15

Ac idi c	88.6	11.4	1. 9 6 8	4.1 12	86. 7	13. 3	0. 6 5 7	2.4 27
Al kal ine	95.1	4.9	1. 9 7 2	4.1 03	89. 1	10. 9	0. 6 1 9	2.4 69
Ox ida tiv e	86.9	13.1	1. 9 5 4	4.1 86	85. 9	14. 1	0. 6 5 4	2.4 85
Re du cti ve	89.3	10.7	1. 9 8 2	4.1 72	89. 0	11. 0	0. 6 0 4	2.4 24
Th er ma l	98.5	1.5	1. 9 1 9	4.1 88	96. 3	3.7	0. 6 4 6	2.4 13
Ph oto lyti c	99.2	0.8	1. 9 4 2	4.1 46	98. 4	1.6	0. 6 1 3	2.4 75
Hy dro lyti c	96.4	3.6	1. 9 0 7	4.1 79	98. 2	1.8	0. 6 9 1	2.4 82

Doxribtimine showed 0.8–13.1% degradation, with maximum degradation under oxidative conditions (13.1%), followed by acidic (11.4%) and reductive (10.7%) conditions. Doxecitine showed 1.6–14.1% degradation, with maximum degradation under oxidative conditions (14.1%), followed by acidic (13.3%) and reductive (11.0%) conditions. The purity angle remained below the purity threshold for both analytes under all stress conditions, confirming peak purity. Thus, the developed RP-HPLC method was specific and stability-indicating for simultaneous determination of both analytes.

8. Conclusion

A stability-indicating RP-HPLC technique was successfully developed and validated for determining doxribtimine and doxecitine in bulk medicinal substances and pharmaceutical dosage forms. The

improved chromatographic conditions resulted in rapid and efficient separation of both analytes while maintaining acceptable retention, peak symmetry, column efficiency, and resolution. The method revealed excellent linearity over the tested concentration range, as well as appropriate accuracy, precision, sensitivity, specificity, and robustness in accordance with the stated validation criteria. The assay results matched the expected medication content, and there was no interference from blank or placebo components. Forced-degradation tests indicated that the approach effectively isolated doxribtimine and doxecitine from their degradation products in a variety of stress settings, including acidic, alkaline, oxidative, reductive, thermal, photolytic, and hydrolytic. The peak-purity results validated the spectral purity of both analyte peaks as well as the method's ability to indicate stability. Overall, the established RP-HPLC method proved to be dependable, reproducible, cost-effective, and appropriate for regular quality-control testing, assay determination, and stability assessment of doxribtimine and doxecitine in pharmaceutical formulations.

9. References

- Garone C, Taylor RW, Nascimento A, Poulton J, Fratter C, Domínguez-González C, et al. Retrospective natural history of thymidine kinase 2 deficiency. *J Med Genet*. 2018;55(8):515–521. doi:10.1136/jmedgenet-2017-105012.
- Wang J, Kim E, Dai H, Stefans V, Vogel H, Al Jasmi F, et al. Clinical and molecular spectrum of thymidine kinase 2-related mitochondrial DNA maintenance defect. *Mol Genet Metab*. 2018;124(2):124–130. doi:10.1016/j.ymgme.2018.04.012.
- US Food and Drug Administration. KYGEVVI (doxecitine and doxribtimine) powder for oral solution: prescribing information. Silver Spring (MD): US Food and Drug Administration; 2025.
- Lopez-Gomez C, Levy RJ, Sanchez-Quintero MJ, Juanola-Falgarona M, Barca E, Garcia-Diaz B, et al. Deoxycytidine and deoxythymidine treatment for thymidine kinase 2 deficiency. *Ann Neurol*. 2017;81(5):641–652. doi:10.1002/ana.24922.
- Lopez-Gomez C, Hewan H, Sirra C, Akman HO, Sanchez-Quintero MJ, Juanola-Falgarona M, et al. Bioavailability and cytosolic kinases modulate response to deoxynucleoside therapy in TK2 deficiency. *EBioMedicine*. 2019;46:356–367. doi:10.1016/j.ebiom.2019.07.037.
- International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH harmonised guideline Q14: analytical procedure development. Geneva: ICH; 2023.
- International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH harmonised guideline Q2(R2): validation of analytical procedures. Geneva: ICH; 2023.
- Mittur A, VanMeter SA, Orujov E, Glidden P. Pharmacokinetics and safety of a 1:1 mixture of doxecitine and doxribtimine: open-label phase 1 single ascending dose and food effect studies in healthy adults. *Clin Ther*. 2024;46(7):576–587. doi:10.1016/j.clinthera.2024.06.006.
- Hernandez-Voth A, Sayas Catalan J, Corral Blanco M, Castaño Mendez A, Martin MA, De Fuenmayor Fernandez de la Hoz C, et al. Deoxynucleoside therapy for respiratory involvement in adult patients with thymidine kinase 2-deficient myopathy. *BMJ Open Respir Res*. 2020;7(1):e000774. doi:10.1136/bmjresp-2020-000774.
- Lopez-Gomez C, Sanchez-Quintero MJ, Lee EJ, Kleiner G, Tadesse S, Xie J, et al. Synergistic deoxynucleoside and gene therapies for thymidine kinase 2 deficiency. *Ann Neurol*. 2021;90(4):640–652. doi:10.1002/ana.26185.
- Berardo A, Domínguez-González C, Engelstad K, Hirano M. Advances in thymidine kinase 2 deficiency: clinical aspects, translational progress, and emerging therapies. *J Neuromuscul Dis*. 2022;9(2):225–235. doi:10.3233/JND-210786.
- Bermejo-Guerrero L, Hernández-Voth A, Serrano-Lorenzo P, Blázquez A, Martin-Jimenez P, Martin MA, et al. Remarkable clinical improvement with oral nucleoside treatment in a patient with adult-onset TK2 deficiency: a case report. *Mitochondrion*. 2024;76:101879. doi:10.1016/j.mito.2024.101879.
- Chow E, Miller L, Clearman A, Arnold P, Koenig MK, Russo SN. Doxecitine and doxribtimine treatment in an adult patient with thymidine kinase 2 deficiency. *Mol Genet Metab*. 2025;145(4):109159. doi:10.1016/j.ymgme.2025.109159.
- U.S. Food and Drug Administration, Center for Drug Evaluation and Research. Integrated review: KYGEVVI (doxecitine and doxribtimine), NDA 219792 [Internet]. Silver Spring (MD): U.S. Food and Drug Administration; 2025.
- European Medicines Agency. Kygevi: EPAR—public assessment report [Internet]. Amsterdam: European Medicines Agency; 2026.
- Talluri Sarada Mrinalini. Computational drug design-in silico revolution reviewed: the evolution, innovation, and influence on modern drug discovery. *BTS International Advanced Pharmaceutical Sciences Journal*. 2026;1(6):39-46.
- Sujana A, Durga Prasad K, Talluri Sarada Mrinalini. Controlled release lercanidipine tablets: a study on formulation and evaluation by wet granulation. *Future J Pharm Health Sci*. 2024;4(2):1-7.

18. Rukhsana S, Vamsi Krishna S, Hasmita K, Ayesha Laharin S, Sri Vani M, Meghamala K, et al. In vivo assessment of acute dermal toxicity and reparative efficacy of phytoconstituent-loaded hydrogel scaffolds incorporating *Calendula officinalis* in full-thickness wound. Int J Environ Sci. 2025;11(23 Suppl):5789-5799.