ORIGINAL ARTICLE

Simultaneous Determination of Doxecitine and Doxribtimine in Bulk Drug and Pharmaceutical Formulation by Reverse-phase Ultra-performance Liquid Chromatographic with Photodiode Array Detection: Method Development and Validation

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Abstract

Objective: The present investigation was undertaken to establish a reliable, sensitive, and efficient reverse-phase ultra-performance liquid chromatographic (RP-UPLC) technique for the concurrent quantification of two pharmacologically significant nucleoside compounds, doxecitine and doxribtimine, in their pure forms and in tablet formulations. **Materials and Methods:** Chromatographic separation was achieved on a phenyl column (100 × 2.1 mm, 1.7 µm particle size) using an isocratic mobile phase of acetonitrile and water (30:70, v/v) buffered to pH 2.5 with formic acid. The system was operated at a flow rate of 0.5 mL/min, and detection was performed at 229 nm with a photodiode array detector. Full validation was carried out in accordance with International Conference on Harmonisation (ICH) Q2(R1) guidelines. **Results:** Both analytes exhibited linearity across the concentration range of 50–300 µg/mL, with correlation coefficients (R^2) of 0.9998 and 0.9996 for doxecitine and doxribtimine, respectively. The limit of detection was 0.40 µg/mL, and the limit of quantification was 1.0 µg/mL for each compound. Recovery, precision, and robustness data were all within ICH-specified acceptance criteria. **Conclusion:** The validated RP-UPLC method presented here offers a fast, cost-effective, and dependable tool for routine quality control of doxecitine and doxribtimine, including stability testing at multiple time points, in both bulk drug substance and finished pharmaceutical products.

Key words: Doxecitine, doxribtimine, International Conference on Harmonisation validation, method development, photodiode array detector, reverse-phase ultra-performance liquid chromatographic

INTRODUCTION

Doxecitine is the synthetic counterpart of deoxycytidine, a naturally occurring pyrimidine deoxyribonucleoside. It occupies a central position in cellular biochemistry as an indispensable constituent of the deoxyribonucleotide pool, which is essential for faithful DNA replication and the repair of damaged genetic material.[1,2] Structurally, it belongs to the pyrimidine family and participates in the nucleotide salvage pathway, providing substrates for both nuclear and mitochondrial DNA (mtDNA) synthesis.

Doxribtimine, commonly referred to as thymidine, is a naturally occurring deoxyribonucleoside that serves as one of the primary building blocks of DNA. Although it exhibits negligible standalone anti-tumor properties, thymidine

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has been the subject of sustained scientific attention over several decades as a biochemical sensitizer capable of enhancing the cytotoxic activity and alleviating the systemic toxicity of established chemotherapeutic agents, including 5-fluorouracil, methotrexate, and cytosine arabinoside (ara-C).[3,4] Its present-day role remains anchored in experimental oncology research and as a modulator within combination chemotherapy regimens.

The fixed-dose combination of doxecitine and doxribtimine, commercially marketed as Kygevvi, constitutes a specific treatment indicated for thymidine kinase 2 (TK2) deficiency – a rare, autosomal recessive mitochondrial disorder.[5] TK2 is a mitochondrial matrix enzyme responsible for phosphorylating pyrimidine deoxyribonucleosides, thereby sustaining the deoxyribonucleotide pools required for mtDNA maintenance.[6,7] Pathogenic variants in the TK2 gene lead to profound mtDNA depletion within post-mitotic tissues, culminating in progressive myopathy, respiratory failure, and, in severe infantile-onset cases, early mortality.[8,9] Conditions characterized by reduced mtDNA copy number, collectively referred to as mitochondrial depletion syndromes, encompass a heterogeneous group of disorders that share this pathological hallmark.[10]

Clinical manifestations associated with TK2 deficiency primarily involve skeletal muscle, reflecting the high energy demands and limited regenerative capacity of post-mitotic myocytes. Patients typically present with progressive proximal muscle weakness and ventilatory compromise requiring mechanical respiratory support.[11,12] The true prevalence of this condition remains unclear; however, with only approximately 120 documented cases in the peer-reviewed literature, it is widely regarded as an ultra-rare disorder, and underdiagnosis owing to phenotypic variability and limited awareness cannot be excluded.[5] The most frequently encountered adverse effects of nucleoside replacement therapy with the Kygevvi combination include gastrointestinal disturbances such as diarrhea and vomiting, transient elevations in hepatic enzyme concentrations, and abdominal discomfort.[13-16]

From an analytical standpoint, the accurate and simultaneous quantification of doxecitine and doxribtimine in pharmaceutical matrices is of direct relevance to quality control, batch release testing, and long-term stability studies. A thorough survey of the published literature identified several analytical methods for the individual determination of these nucleosides; however, no validated chromatographic procedure for their concurrent estimation in a combined pharmaceutical formulation has been described. Reported high-performance liquid chromatography (HPLC) methods for doxecitine typically employ C18 columns with phosphate or acetate buffer systems, run times exceeding 10 min, and ultraviolet (UV) detection in the range of 254–270 nm. Similarly, thymidine (doxribtimine) has been quantified by reversed-phase HPLC on C18 stationary phases using methanol–water mobile phases, with run times of 8–15 min. These conventional platforms, while validated individually, present limitations including prolonged analysis times, incompatibility with high-throughput batch testing, and the absence of simultaneous multi-analyte capability within a single injection. The present study addressed these gaps by developing a sub-3-min reverse-phase ultra-performance liquid chromatographic (RP-UPLC) method on a phenyl column capable of resolving both analytes in a single run, thereby offering a clear advantage in terms of speed, solvent consumption, and throughput over existing individual HPLC procedures. The method was fully validated in accordance with International Conference on Harmonisation (ICH) Q2(R1) harmonised guidelines. The chemical structures of doxecitine and doxribtimine are depicted in Figure 1.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (HPLC grade) and formic acid (analytical reagent grade) were procured from Merck India Ltd., Mumbai, India. HPLC-grade water was prepared in-house using a Milli-Q purification system (Millipore, USA). Working standards of doxecitine and doxribtimine were generously gifted by Zydus Cadila, Ahmedabad, India, with certificates of analysis confirming purity ≥99.5% for each substance.

Instrumentation

All chromatographic experiments were conducted on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled to a photodiode array (PDA) detector (model 2998). The system was interfaced with Empower 2.0 data management software for instrument control and chromatogram acquisition.[17,18] Analytical weighing was performed using a Sartorius analytical balance (d = 0.01 mg), and solution sonication was carried out in an ultrasonic bath operating at 40 kHz and 360 W.

Method optimization

Preliminary scouting experiments were conducted to identify column chemistry and mobile phase composition best suited

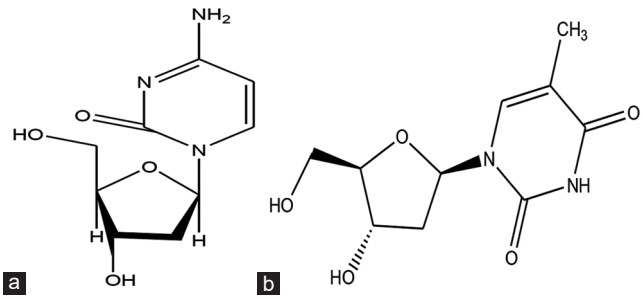

Figure 1: Chemical structures of (a) doxecitine and (b) doxribtimine

to the simultaneous elution of the two analytes with adequate resolution and acceptable run time. Reversed-phase columns evaluated included a C18 column and a phenyl column of identical dimensions. Mobile phase combinations were screened at various proportions of acetonitrile and formic-acid-buffered water (pH 2.5).

Optimal chromatographic performance was achieved using the phenyl column (100 × 2.1 mm, 1.7 µm; Waters BEH Phenyl), an isocratic mobile phase of acetonitrile and pH 2.5 formic acid buffer in a ratio of 30:70 (v/v), a flow rate of 0.5 mL/min, a column maintained at ambient temperature, and an injection volume of 5 µL. Under these conditions, doxecitine eluted at approximately 1.143 min and doxribtimine at approximately 2.422 min. The two peaks were resolved with a resolution factor of 6.48, comfortably exceeding the USP threshold of 2.0.

Selection of detection wavelength

UV spectra of doxecitine and doxribtimine were recorded individually over the wavelength range 200–400 nm. An isobestic point – the wavelength at which both compounds exhibit identical molar absorptivities – was identified at 229 nm. Selection of this wavelength as the detection point ensured equitable and accurate quantification of both analytes from a single chromatographic run without any requirement for spectral correction factors.

Preparation of buffer

One liter of HPLC-grade water was acidified with an appropriate quantity of formic acid until the pH electrode reading stabilized at 2.5 (±0.05). The resulting buffer solution was vacuum-filtered through a 0.22 µm cellulose nitrate membrane filter before use as the aqueous component of the mobile phase.

Preparation of standard solutions

Accurately weighed quantities (20 mg each) of the doxecitine and doxribtimine working standards were transferred into a 10 mL volumetric flask. Approximately 7 mL of diluent (HPLC water) was added, and the mixture was sonicated at 40 kHz for 10 min to achieve complete dissolution. The volume was then adjusted to the 10 mL graduation mark with diluent to furnish a stock concentration of 2000 µg/mL for each analyte. Working standard solutions at 200 µg/mL were obtained by transferring 1.0 mL of the stock into a fresh 10 mL flask and diluting to volume with diluent.

Preparation of sample solutions

Forty milligrams of finely ground tablet powder was accurately weighed into a 10 mL volumetric flask. Diluent (7 mL) was added, and the mixture was ultrasonicated for 20 min to facilitate the complete extraction of both active constituents. After cooling to room temperature, the volume was made up to the mark. An aliquot of 1.0 mL from this preparation was pipetted into a separate 10 mL flask and diluted with diluent to yield a nominal concentration of 200 µg/mL for each analyte. Before injection, the sample solution was filtered through a 0.22 µm syringe filter to remove particulate matter.

Validation procedure

The method was validated in strict accordance with ICH Q2(R1) guidelines covering system suitability, specificity, linearity, accuracy, precision (intraday and interday), limits of detection and quantification, robustness, and forced degradation studies.[19,20]

RESULTS AND DISCUSSION

System suitability

Before commencing formal validation, the suitability of the system was evaluated by injecting six replicate injections of the working standard solution (200 µg/mL each). Parameters assessed included USP plate count, tailing factor, resolution between the two analyte peaks, and retention time reproducibility. The results, summarized in Table 1, confirmed that the chromatographic system was functioning within acceptable boundaries before sample analysis.

Specificity

Specificity was assessed by comparing chromatograms of the blank diluent, a placebo mixture (composed of all tablet excipients but lacking active drug), and the standard solution. No chromatographic peaks attributable to formulation excipients or diluent components appeared in the retention-time windows of either analyte. Complete resolution of doxecitine and doxribtimine from each other and from all matrix components was achieved, confirming the selectivity of the method for its intended purpose [Figures 2 and 3].

Linearity

Linearity was assessed across six concentration levels spanning 25–150% of the nominal working concentration (200 µg/mL), yielding final concentrations of 50, 100, 150, 200, 250, and 300 µg/mL for both doxecitine and doxribtimine. Peak areas were plotted against the respective concentrations to construct the calibration curves. Both compounds demonstrated excellent linear relationships over this range, with correlation coefficients of 0.99984 and 0.99955 for doxecitine and doxribtimine, respectively [Table 2 and Figure 4]. The

Table 1: System suitability parameters (Mean±SD; *n*=6)

Parameter	Doxecitine		Doxribtimine	
	Mean	Standard deviation	Mean	Standard deviation
USP plate count	10852	0.00352	10235	0.00857
USP tailing factor	0.92	0.00659	1.04	0.01854
USP resolution	-	-	6.48	0.00326
Retention time (min)	1.148	0.00154	2.431	0.00298

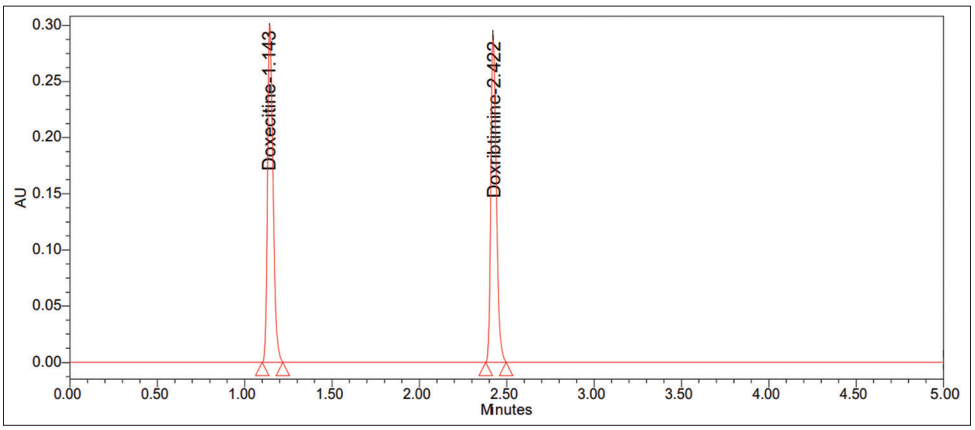

Figure 2: Representative chromatogram of the standard solution

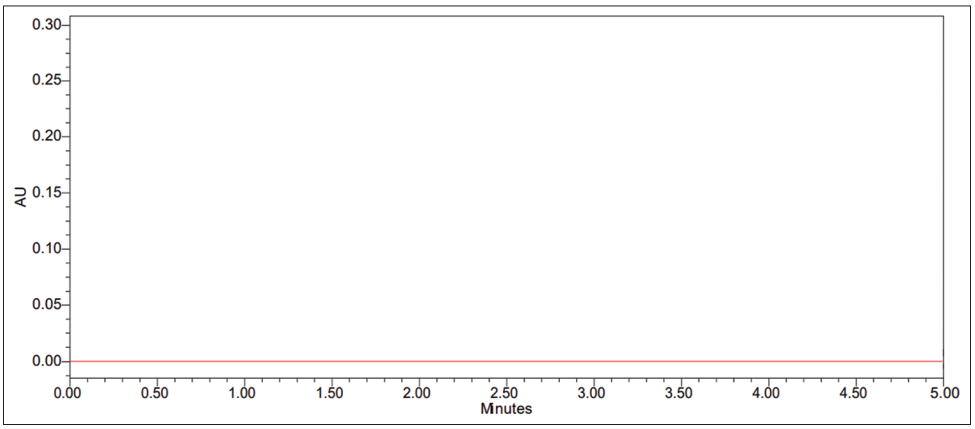

Figure 3: Representative chromatogram of Blank

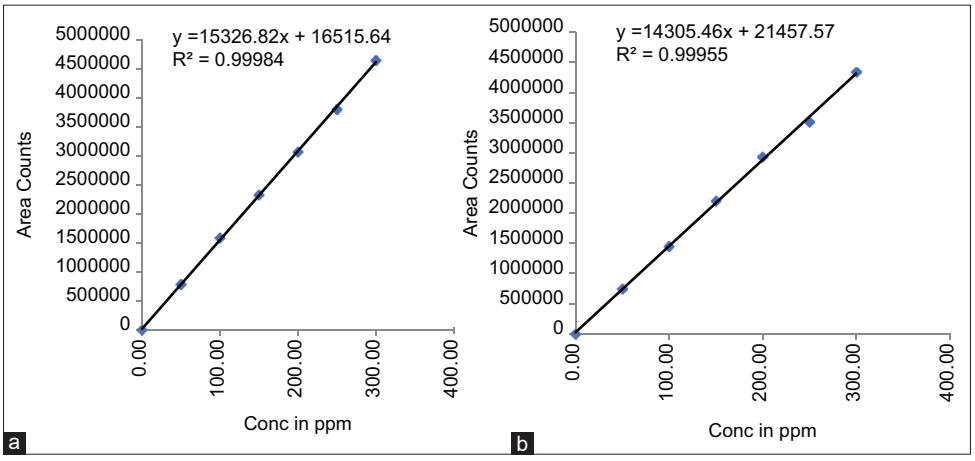

Figure 4: Calibration plot of (a) doxecitine and (b) doxribtimine

standard error of the slope was 98.4 for doxecitine and 112.7 for doxribtimine, whereas the standard error of the intercept was 17,342 and 19,816, respectively. Residual analysis confirmed that the individual data points were randomly scattered around the regression line with no systematic trend, indicating homoscedasticity across the calibrated range. The 95% confidence intervals for the slopes were 15,326.82 ± 232.1 (doxecitine) and 14,305.46 ± 266.3 (doxribtimine). The regression equations obtained were consistent with passing through or close to the origin, indicating negligible systematic bias across the calibrated range.

Accuracy

To evaluate the accuracy of the proposed method, three concentration levels corresponding to 80, 100, and 120% of the nominal working concentration were prepared and analyzed in triplicate. The mean percentage recoveries obtained at all three levels were within the ICH-specified acceptance range, confirming that the method was both accurate and reliable. Recovery data are presented in Table 3.

Precision

Precision was evaluated at two levels: intraday (repeatability) and interday (intermediate precision). For intraday precision, six individual sample preparations at 200 µg/mL were prepared and analyzed within a single analytical session on the same instrument. Interday precision was established by repeating the analysis on a different calendar day using an alternative UPLC system (Agilent 1290 Infinity UPLC) by a different analyst within the same facility, in accordance with ICH intermediate precision requirements.

All six replicate measurements for both intraday and interday studies yielded %assay values falling within the 98–102% acceptance window. The relative standard deviation % (RSD) for intraday precision was 0.93% for doxecitine and 0.77% for doxribtimine, whereas interday precision produced %RSD values of 0.71% and 0.86%, respectively [Tables 4 and 5]. These findings confirm that the method delivers reproducible and consistent results regardless of minor day-to-day and instrument-to-instrument variability [Figure 5].

Limit of detection (LOD) and limit of quantification (LOQ)

The signal-to-noise (S/N) ratio approach was employed to establish the LOD and LOQ for both analytes. Serial dilutions of the standard solution were prepared and injected; LOD was defined as the lowest concentration producing an S/N ratio of at least 3:1, and LOQ was defined as the concentration yielding an S/N ratio of not <10:1. For doxecitine and doxribtimine alike, the LOD was determined to be 0.40 µg/mL and the LOQ 1.0 µg/mL, as presented in Table 6. These figures underscore the high sensitivity of the proposed method, rendering it suitable for the detection of trace-level impurities or degradation products.

Table 2: Linearity data for doxecitine and doxribtimine

S.No	Doxecitine		Doxribtimine	
	Conc. (µg/mL)	Area count	Conc. (µg/mL)	Area count
1	50.00	784512	50.00	742316
2	100.00	1584623	100.00	1447542
3	150.00	2325568	150.00	2201320
4	200.00	3065234	200.00	2935264
5	250.00	3800542	250.00	3508923
6	300.00	4648293	300.00	4335568
Correl coeff		0.99984		0.99955
Slope		15326.82		14305.46
Intercept		16515.64		21457.57

Table 3: Accuracy results (Mean±SD; *n*=3)

S. No	% Level	Doxecitine			Doxribtimine		
		Mean % Recovery	Standard deviation	% RSD	Mean % recovery	Standard deviation	% RSD
1	50	99.3	0.273	0.27	98.9	0.310	0.31
2	100	99.6	0.365	0.37	98.7	0.358	0.36
3	150	99.4	0.446	0.45	99.9	0.255	0.26

RSD: Relative standard deviation

Table 4: Intraday precision results (Mean±SD; *n*=6)

S.No	Doxecitine			Doxribtimine		
	Conc. (µg/mL)	Area counts	% Assay	Conc. (µg/mL)	Area counts	% assay
1	200	3052634	100.5	200	2945821	99.1
2		3014278	99.3		2965745	99.8
3		3056982	100.7		2995231	100.8
4		3032364	99.9		2932105	98.7
5		3078452	101.4		2978452	100.2
6		3002145	98.9		2953201	99.4
% RSD			0.93	% RSD		0.77
Mean			100.1	Mean		99.7
SD			0.9304	SD		0.7633

RSD: Relative standard deviation

Table 5: Interday precision results (Mean±SD; *n*=6)

S. No	Doxecitine			Doxribtimine		
	Conc.(µg/mL)	Area counts	% assay	Conc.(µg/mL)	Area counts	% assay
1	200	3045278	100.3	200	2924523	98.4
2		3012563	99.2		2978452	100.2
3		3020231	99.4		2965321	99.7
4		3052643	100.5		2950263	99.2
5		3058264	100.7		2946536	99.1
6		3011245	99.1		2998542	100.8
% RSD			0.71	% RSD		0.86
Mean			99.9	Mean		99.6
SD			0.7118	SD		0.8548

RSD: Relative standard deviation

Table 6: LOD and LOQ for doxecitine and doxribtimine

Doxecitine				Doxribtimine			
LOD		LOQ		LOD		LOQ	
Concentration	s/n	Concentration	s/n	Concentration	s/n	Concentration	s/n
0.40 µg/mL	3	1.0 µg/mL	10	0.40 µg/mL	3	1.0 µg/mL	10

LOD: Limit of detection, LOQ: Limit of quantification

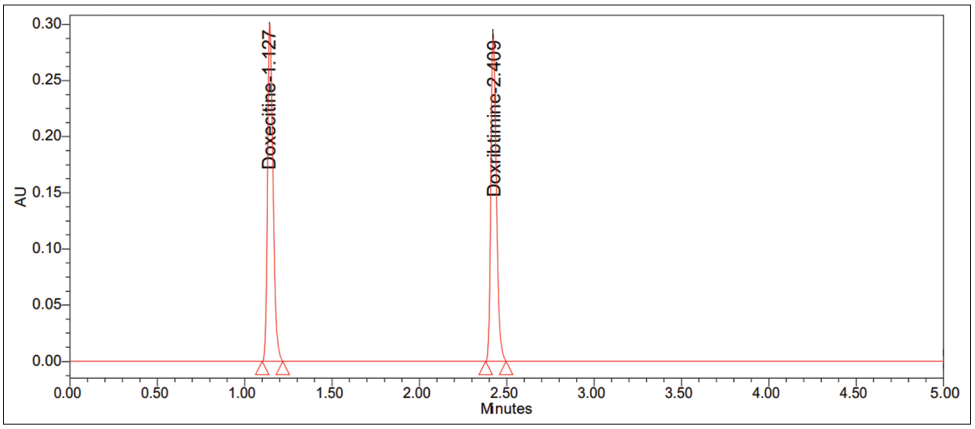

Figure 5: Chromatogram obtained during method precision evaluation

Table 7: Robustness data (Mean±SD; *n*=3)

Parameter name	Doxecitine			Doxribtimine		
	Mean	SD	%RSD	Mean	SD	%RSD
Flow minus (0.45 mL/min)	99.6	0.702	0.70	99.8	0.721	0.72
Flow plus (0.55 mL/min)	100.5	0.95	0.95	99.8	0.379	0.38
Organic minus (27:73)	99.1	0.643	0.65	99.8	0.346	0.35
Organic plus (33:67)	99.5	0.600	0.60	99.5	0.945	0.95

RSD: Relative standard deviation, values expressed as Mean±SD (*n*=3)

Robustness

Robustness was investigated by deliberately introducing minor perturbations to the principal chromatographic parameters – namely, mobile phase flow rate and organic modifier ratio – while keeping all other conditions unchanged. Flow rate was varied between 0.45 and 0.55 mL/min (nominal 0.50 mL/min), and the acetonitrile fraction was adjusted to 27:73 and 33:67 (v/v) relative to the optimal 30:70 ratio. In all instances, mean %assay values remained within 99.1–100.5%, and %RSD values stayed below 1.0% [Table 7]. One-way Analysis of Variance applied to the %assay results across the four perturbed conditions revealed no statistically significant difference from the nominal condition ($F = 1.24$, $P > 0.05$), confirming that the observed variation falls within routine analytical noise rather than reflecting a true method performance change. These findings collectively demonstrate that the method maintains its performance characteristics despite small, practically encountered variations in operating parameters.

Forced degradation studies

Stress testing experiments were conducted to evaluate the inherent stability of both analytes under a range of physicochemical degradation conditions and to demonstrate the ability of the method to separate the analyte peaks from any degradation products, thereby confirming specificity in a stressed sample environment.

Acid degradation

Forty milligrams of the sample was treated with 1 mL of 1 N hydrochloric acid in a 10 mL volumetric flask. After standing undisturbed at room temperature for 60 min, the solution was neutralized by the addition of 1 mL of 1 N sodium hydroxide, brought to volume with diluent, and diluted further as required before filtration and injection.

Alkali degradation

Sample (40 mg) was exposed to 1 mL of 1 N NaOH for 60 min, after which 1 mL of 1 N HCl was added to neutralize the mixture. The solution was then diluted and processed in the same manner as the acid-stressed samples.

Table 8: Forced degradation results

Degradation condition	Doxecitine		Doxribtimine	
	% assay	% Deg	% assay	% Deg
Control degradation	100	0	99.9	0.1
Acid degradation	87.9	12.1	99.3	0.6
Alkali degradation	88.4	11.6	86.2	13.7
Peroxide degradation	86.4	13.6	88	11.9
Reduction degradation	95.3	4.7	98.7	1.2
Thermal degradation	96.5	3.5	89.7	10.2
Photolytic degradation	89.2	10.8	97.4	2.5
Hydrolysis degradation	90.8	9.2	96.1	3.8

Oxidative degradation

Forty milligrams of the sample was treated with 1 mL of 10% hydrogen peroxide and held at room temperature for 60 min, after which the solution was diluted, filtered, and analyzed.

Reductive degradation

The sample was subjected to 1 mL of 10% sodium bisulfite solution for a period of 60 min before dilution and analysis.

Thermal degradation

Fifty milligrams of standard material was spread in a Petri dish and placed in a hot-air oven at 105°C for 6 h. Following heat exposure, the degraded material was reconstituted gravimetrically (20 mg equivalent), dissolved in diluent, diluted to the working concentration, and injected.

Photolytic degradation

One hundred milligrams of the sample was exposed to a photostability chamber under conditions compliant with ICH Q1B (visible light $\geq 1.2 \times 10^6$ lux·h and near-UV $\geq$ 200 W·h/m^2) for 6 h. The stressed material was then dissolved and diluted customarily.

Hydrolytic degradation

Forty milligrams of the sample was mixed with 1 mL of HPLC water in a 10 mL volumetric flask, allowed to stand for 60 min at room temperature, diluted to volume, and filtered before injection.

The percentage degradation observed under each condition is compiled in Table 8. Doxecitine proved most susceptible to acidic conditions (12.1% degradation) and peroxide treatment (13.6% degradation), whereas doxribtimine showed greatest instability under alkaline stress (13.7% degradation) and peroxide exposure (11.9% degradation). Both analytes were comparatively stable under reductive, thermal, and photolytic conditions. Notably, all degradation peaks were well-resolved from the principal analyte peaks, further substantiating the specificity of the validated method for stability-indicating analyses.

CONCLUSION

A simple, rapid, and stability-indicating RP-UPLC method employing a PDA detection at 229 nm has been developed and rigorously validated for the concurrent determination of doxecitine and doxribtimine. Separation was achieved on a phenyl column within a total run time of approximately 3 min using an isocratic mobile phase, which makes the procedure both time-efficient and economical for high-throughput quality control laboratories. All ICH Q2(R1) validation parameters – including specificity, linearity, accuracy, intraday and interday precision, robustness, and sensitivity – were satisfactorily demonstrated, with %RSD values for all precision studies remaining below 2.0%. The forced degradation experiments confirmed the stability-indicating capability of the method, as each analyte peak was adequately resolved from its respective degradation products under all stress conditions examined. Taken together, the method can be reliably implemented for routine quality assurance testing, batch release analysis, and stability monitoring of pharmaceutical dosage forms containing the doxecitine–doxribtimine fixed-dose combination.

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AUTHORS' CONTRIBUTIONS

Ch. Ravi contributed to the literature review, experimental design, sample preparation, data collection, statistical analysis, and drafting of the original manuscript. Dr K. Kiran Kumar participated in the conceptualization of the study, the critical review of the manuscript, the supervision of laboratory work, and the oversight of the overall research execution. Dr. G. Krishnaveni was responsible for reviewing and editing the manuscript, inspecting experimental results, and providing critical scientific commentary on the analytical findings.

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