A New Reverse Phase High-Performance Liquid Chromatography (RP- HPLC) Method for Determination of Kinetin Riboside (plant hormone) in Dequalinium Chloride Based Self-Assembled Vesicles: Development, Validation, and Force Degradation Study

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Abstract

The analytical procedure remains the fundamental part of formulation development in terms of quantification of the drug in the formulation. The purpose of the current study was to develop accurate, sensitive and rapid high performance liquid chromatography (RP-HPLC) method for the quantitation of kinetin riboside (KR) in KR-loaded dequalinium based vesicles, also known as DQAsomes. The chromatographic analysis was performed on C_{18} column with detection wavelength of 269 nm for KR and 310 nm for dequalinium chloride (DQA). The mobile phase consisted of 0.05 M potassium phosphate buffer and methanol in gradient system. The method was further validated according to the guidelines set by ICH. The developed chromatographic method was found to be linear from 10 µg/mL to 250 µg/mL with r^2 value of 0.9995. All other validation parameters including accuracy, precision, robustness, etc. were well within the acceptable limits. The current research also encompasses force degradation studies to understand the impact of different stressing conditions on the stability of drugs in the formulation. KR-loaded DQAsomes were formulated by modified film hydration method and characterized for particle size (215.32±3.54nm), size distribution (0.12±0.004) and zeta potential (39.4±3.04mV). The developed and validated method was further applied to evaluate KR entrapment and loading from KR-loaded DQAsomes along with *in vitro* cumulative KR release from the formulation. This research would further act as a foundation for the quantification of the drug in other dequalinium-containing formulations.

Introduction

Kinetin riboside (KR) **(**Fig. 1**(A))** is a cytokinin derived from plants that is composed of 2-(6-(furan-2-ylmethylamino) purin-9-yl)-5-(hydroxymethyl) oxolane-3,4-diol. KR is known to elicit anticancer effects through downregulation of Bcl-2 and upregulation of Bax. Cytochrome C levels also rise in tandem with procaspase-9 activity, with no effect on caspase-8, highlighting its activity only on targeted cancer cells [1]. The KR was found to be associated with apoptotic death of HeLa cells and mouse melanoma B16F-10 cells. Through the mitochondrial apoptosis pathway, KR upregulates caspase-3, Bad, and downregulates Bcl-2 and releases cytochrome C by disrupting the mitochondrial membrane [2].

In the recent past, novel drug delivery systems have grabbed the attention of formulation scientists for their dual purpose of targeting delivery to cancer cells along with improved therapeutic efficiency [3, 4]. Several reports suggest that mitochondria, chief organelles for energy production, plays an important role in regulating redox potential, glutathione levels, calcium levels, and the apoptotic process [5–9]. Lately, mitochondrial drug delivery has become popular, especially in the area of cancer therapeutics [10, 11]. Triphenyl phosphonium, rhodamine 19, rhodamine 123, and dequalinium are some of the examples of mitochondrial targeting molecules [12]. Dequalinium chloride (DQA) (Fig. 1**(B)**), a bola-amphiphilic molecule, is known to possess two hydrophilic groups at both ends connected by a hydrophobic alkyl chain [13]. It is primarily an antimicrobial (bactericidal and fungicidal) agent. However, its use has been extended to other therapeutic areas as well. It exerts mitochondrial targeting activity due to its positively charged nature, and it has a propensity towards the higher negative mitochondrial membrane potential of

cancerous cells, thereby showing greater accumulation. The DQA downregulates mRNA expression levels of genes (NFKB2, HRAS, NF1, CBL, RAF1, and Bcl-2), and upregulates the level of caspase-3 (apoptotic protease), thereby facilitating programmed cell death [14].

Weissig found that dequalinium has the property of getting self-assembled into colloidal vesicles which are called DQAsomes [15]. Weissig and co-workers also suggested that DQAsomes can bind plasmid DNA (pDNA) and other drugs to elicit targeted drug delivery [16].

The quantification process is an integral part of formulation development. Ge et al. developed a sensitive LC/MS/MS method for the identification and quantitation of KR in coconut water [17]. Our literature search has revealed no reports regarding quantitation of KR in KR-loaded DQAsomes. Thus, it is an unfulfilled need to have a reliable, robust, and precise RP-HPLC method for the determination of KR content in DQAsomes.

The present work focuses on developing a sensitive and accurate RP-HPLC method for the quantification of KR in DQAsomes. The method was further validated as per ICH Q2 (R1) and checked for accuracy, linearity, precision, robustness, and other parameters [18]. The three batches of KR-loaded DQAsomes were formulated by varying one of the impacting processing parameters, probe sonication amplitude. The formulated batches were characterized for particle size, polydispersity index and zeta potential. The validated method can be utilized for the evaluation of KR-loaded DQAsomes for estimating entrapment efficiency, drug loading and KR *in vitro* release. The force degradation study was also done to find out the impact of different stress conditions on the drug as well as on the formulation.

Materials And Methods

Chemicals and reagents

Kinetin riboside (%purity,>98%w/w), dequalinium chloride (%purity,>95%w/w) and potassium dihydrogen phosphate were obtained from Sigma Aldrich (Mumbai, India). HEPES buffer was obtained from HiMedia labs (Pennsylvania, USA). Methanol of HPLC grade was purchased from Fisher chemicals. All other chemicals were purchased from Merck limited, Germany. The filtration of the mobile phase (buffers and solvents) used in HPLC experimentation was done by 0.22µm membrane filter (Millipore) and then sonicated in bath sonicator for 30 minutes.

Instrumentation and RP-HPLC conditions

The chromatographic system comprised of the quaternary pump (DEAEU02393) with a photo-diode array as detector (DEAC614415) fitted with an auto-sampler (DEAEQ40884) and data acquisition software (Open Lab). For separation purposes, a reversed phase-HPLC column (XBridge C_{18}, 150mm×4.6mm, 5µ) was used. The column was thoroughly washed and later equilibrated for at least 30 min prior to analysis. The chromatographic data was obtained using Open Lab software.

The gradient system was employed for the separation of KR and DQA. The mobile phase was comprised of 0.05 M potassium dihydrogen phosphate buffer (pH 3.2, adjusted with ortho-phosphoric acid) and methanol as an organic solvent. The composition of the mobile phase varied with respect to time, which has been given in **Table 1**. The flow rate of the mobile phase was kept at 1 mL min^{-1} throughout the run. The column oven temperature was set to be 30°C and the injection volume was set as 10 µL. All the standard and sample solutions were prepared in methanol. The run time was set to be 15 min at λ_{max} of 269nm for KR and at λ_{max} of 310 nm for DQA.

Table 1. HPLC method development-Mobile phase gradient system

A: 0.01 M KH_2PO_4(pH 3.2, adjusted with ortho-phosphoric acid); B: Methanol

Time (min)	A composition	B composition
0	80	20
3	50	50
6	30	70
7	10	90
9	0	100
12	0	100
13	80	20
15	80	20

Preparation of standard and sample stock solutions

A standard stock solution of 1000 µg/mL of KR and DQA was prepared by accurately weighing 10 mg of KR and 10 mg DQA and then dissolved in 10 mL methanol. Further dilutions were prepared from the same standard stock solution of 1000 µg/mL to prepare different solutions of KR and DQA (10, 20, 50, 100, 150, 200, and 250 µg/mL). All the solutions were stored in a refrigerator having a temperature of 2–8°C.

Validation of RP-HPLC method

The validation of the RP-HPLC method for KR and DQA was performed following ICH guidelines Q2 (R1); 'Validation of analytical procedures' [18]. The analytical parameters like 'specificity, system suitability, linearity, precision, accuracy, robustness, the limit of detection (LOD), and the limit of quantitation (LOQ)' were assessed by following the given guidelines [19].

Specificity

Specificity can be defined as “the ability to evaluate the analyte in the presence of other substances like impurities, excipients, or degradants present in the system”. It gives insights into the method’s suitability for the measurement of drugs without interference from excipients. [20]. In this research work, the excipient in the formulation includes HEPES buffer. The samples of DQA and HEPES were spiked with standard KR solution (100 µg/mL) to determine specificity.

System suitability

Six replicates of KR and DQA solutions, each having a concentration of 100 µg/mL were injected to determine system suitability. The number of theoretical plates, tailing factor, and % relative standard deviation (% RSD) were determined and the acceptance criterion for all aforementioned parameters was set to be not more than 2% [21].

Linearity

Linearity represents the ability to get test results that are in direct proportion to the concentration of samples. The different concentrations of KR and DQA (10, 20, 50, 100, 150, 200, and 250 µg/mL) were prepared and injected into RP-HPLC for the determination of linearity. The solutions were injected in triplicate. The calibration curve of peak responses against respective concentration was plotted and regression analysis was done to calculate the coefficient of correlation (r^2) and line equation (slope and Y-intercept) [22]. The limit of r^2 should be greater than 0.999 set by the guideline.

Accuracy

Accuracy represents the proximity of agreement between the accepted value which is generally regarded as a reference and the obtained value. For determination of accuracy, three different solutions of KR and DQA having concentrations of 50 µg/mL, 100 µg/mL, and 150 µg/mL were prepared. All solutions were prepared in triplicate for each concentration. The results were reported in terms of % recovery using the following equation [23].

$$\%Recovery = \frac{Recoveredconcentration}{Injectedconcentration} \times 100 \quad \textbf{(Eq. 1)}$$

The acceptance limit for % recovery should be within the range of 98–102%.

Precision

The precision of the analytical method denotes “the closeness of agreement between injections of multiple samples taken from the same solution under prescribed conditions” [24]. In this research work, method precision was carried out. Six replicates of standard solutions of combined KR and DQA having concentrations of 100 µg/mL were taken and analyzed by RP-HPLC on two consecutive days. The precision was determined by calculating % RSD. The acceptance limit for % RSD was less than or equal to 2%.

Limit of detection (LOD)

Limit of detection (LOD) can be defined as “the lowest possible concentration of analyte which can be detected but is not necessarily quantifiable under the given experimental conditions”. It is computed by the below equation:

$$LOD = \frac{SD}{S} \times 3.3 \quad \textbf{(Eq. 2)}$$

Where SD represents the standard deviation of analyte response (Peak area) and S represents the slope of the calibration curve obtained [22].

Limit of quantification (LOQ)

The limit of quantification represents “the lowest concentration of analyte which can be accurately and precisely quantified”. It is computed by the following equation:

$$LOQ = \frac{SD}{S} \times 10 \quad \textbf{(Eq. 3)}$$

Where SD represents the standard deviation of analyte responses and S represents the slope of the calibration curve obtained while determining linearity [22].

Robustness

The robustness is “the capacity of the method to remain unaffected by little but intentional changes in the method to ensure the reliability of method”. In this study, the following variations have been done to the method.

i. Change in flow rate: 0.8, 1.0, and 1.2 mL/min
ii. Change in column oven temperature: Three temperatures were taken into consideration, viz; 25˚C, 30˚C, and 35˚C
iii. Change in wavelength: Scanning of peaks at 267, 269, and 271nm
iv. Change in mobile phase (Buffer: Methanol) composition: 78:22 (v/v), 80:20 (v/v) and 82:18 (v/v)

The analysis was done in triplicate and the impact of these variables was seen on retention time, peak area, and % recovery [25].

Forced degradation study

The forced degradation study was carried out to assess the effects of varied stress conditions like acidic, basic, thermal, oxidative, and photolytic conditions on the stability of KR and DQA. The force degradation study was done on HEPES buffer (blank), KR, DQAsomes without KR (placebo), and KR-loaded DQAsomes. Briefly, drug solutions were prepared (1000 µg/mL) and were subjected to the aforementioned stress conditions. For acid-base degradation, drug solutions of KR and DQA were dispersed in 1N HCl and 1N NaOH at 60˚C for 48 hours, respectively. After cooling to room temperature,

solutions were neutralized with HCl or NaOH and then diluted to 100 µg/mL with methanol for injecting into RP-HPLC. Hydrogen peroxide of 30% (1mL) was added to drug solutions (1000 µg/mL; 1mL) and kept at room temperature for 7 days to see any oxidative degradation. It was then diluted to 100 µg/mL with methanol for injection into RP-HPLC. For thermal degradation, samples were kept at 80˚C for 48 hours. After 48 hours, all samples were cooled down to room temperature and further diluted to 100 µg/mL with methanol for chromatographic analysis by RP-HPLC. Hydrolytic degradation was assessed by dispersing drug samples with water (1 mL) at room temperature and kept for 24 hours. Photolytic degradation was also carried out by exposing the formulation to UV light (200 W-h/m^2) for one UV cycle (27 hours) in a photostability chamber maintained at 40˚C and 75% RH. The samples were analyzed by the validated RP-HPLC method after making dilutions with methanol to 100 µg/mL [26, 27]. The chromatograms of blank (HEPES), KR, placebo formulation (devoid of KR), and KR-loaded formulation were later evaluated for peaks other than the peak for the drug.

Applications of RP-HPLC method

The RP-HPLC method which has been validated, was employed to calculate the estimation of KR in formulated DQAsomes. The DQAsomes were further characterized for size of particles, dispersity index, and zeta potential. The %entrapment efficiency and %drug loading were evaluated for KR in formulated KR-loaded DQAsomes.

Formulation of KR-loaded DQAsomes

KR-loaded DQAsomes were formulated by the modified film hydration method [16, 28, 29]. In brief, dequalinium chloride (39 mg, 15 mM) and KR (70.5 mg, 15 mM) were dissolved in 7 mL of methanol in a round flask. It was then evaporated with the help of a rotary evaporator (IKA, RV10). The rotational speed was set to be 100 RPM and the temperature was maintained at 37˚C. The thin film was obtained after 15 minutes of stirring and the methanol was completely evaporated. The HEPES buffer (5 mM, pH 7.4) was then added to the round bottom flask to hydrate the formed film, and it was further rotated for 15 min. The obtained suspension was probe sonicated for 1 h at different amplitudes, viz., 30A, 50A, and 70A, so as to have 3 formulation batches. In order to get rid of any undissolved matter in the suspension, it was centrifuged for 20 min at 3000 RPM, and thus, KR-loaded DQAsomes were obtained.

Characterization of formulated KR-loaded DQAsomes

The KR-loaded DQAsomes were formulated by the aforementioned method. The characterization study was done for its particle size, poly dispersibility index (PDI), and zeta potential with the help of the zeta sizer (Malvern ZS, UK). All the measurements were taken at 25˚C.

Evaluation of %entrapment efficiency (%EE) and %drug loading

The amount of KR inside DQAsomes was determined by the centrifugation method [30, 31]. Briefly, the formed suspensions of KR-loaded DQAsomes were centrifuged and rotated at 14000 RPM for 30

minutes. The pellet was formed at the bottom. The supernatant was collected and analyzed by the validated RP-HPLC method, and the entrapment efficiency and drug loading of KR were calculated using the following formula:

$$\%EE = \frac{Amount of kinetin riboside (KR) in DQAsomes}{Initial amount of KR incorporated in DQAsomes} \times 100 \quad \textbf{(Eq. 4)}$$

$$\%Drug Loading = \frac{Amount of kinetin riboside (KR) in DQAsomes}{Weight of nanoparticles} \times 100 \quad \textbf{(Eq. 5)}$$

In vitro drug release

In vitro drug release from DQAsomes was determined by the dialysis bag membrane method [19]. The dialysis bag membrane (MWCO 14 kDa, HiMedia lab, Mumbai) was soaked in pH 6.5 phosphate buffer at least for 24 hours. The KR-loaded DQAsomes of 1 mL equivalent to 10 mg of KR were incorporated into the dialysis bag and the bag was sealed at both ends. It was then suspended in 50 mL of pH 6.5 phosphate buffer and put on a magnetic stirrer at 100 RPM at 37˚C. 1 mL of sample was withdrawn at each time point (0.25,0.5,1,2,4,8,12 and 24h) and was replaced with 1mL fresh dissolution media. The samples were diluted for further chromatographic analysis.

Results And Discussion

Development of RP-HPLC method

The simultaneous determination of KR and DQA was done with the development of the RP-HPLC method. The mobile phase was a mixture of components A and B wherein, A corresponded to 0.01 M potassium phosphate buffer of pH 3.2 and B had methanol as an organic phase. The gradient program was run and the varied composition with respect to time is represented in **Table 1**. The reversed-phase C_{18} X Bridge column (150×4.6 mm, 5µm) was used to get a sharp, narrow, and symmetrical shape. Better separation of KR and DQA was obtained with a flow rate of 1 mL/min and column oven temperature of 30˚C **(Fig. 2)**.

Validation of RP-HPLC method

Specificity

Specificity was determined after comparison of the chromatograms of KR, blank DQAsomes without KR (placebo), and KR-loaded DQAsomes. KR in pure form and in the formulation showed similar retention time indicating no interference of other peaks. It has been represented in **Fig. 3**.

System suitability

The system suitability was evaluated after six replicate injections into the system for a solution of 100 µg/mL for both the analytes, KR and DQA. The %RSD value was found to be well within 2% for different

parameters like peak area, tailing factor, and theoretical plates. **Supplementary Table 1 (Table S1)** represents system suitability parameters with average, standard deviation, and % RSD values.

Linearity

The linearity was established from 10 µg/mL to 250 µg/mL for both KR and DQA. The calibration curve was plotted for peak area against its respective concentration. The equation, Y= 30.155X-49.011 with r^2 value of 0.9995 was obtained for KR while Y= 15.175X-37.774 with r^2 value of 0.9995 was obtained for DQA which represented a good correlation **(Supplementary Fig. S1). Supplementary Table 2** (**Table S2**) represents the linearity study of KR and DQA.

Accuracy

The accuracy was evaluated in terms of % recovery for 50 µg/mL, 100 µg/mL, and 150 µg/mL solutions for the analytes, KR and DQA. It was found that % the recovery was from 99% to 101% for KR and 98 to 100% for DQA. The results are incorporated into **Table 2**. The values were within the range of 98-102% and % RSD was also less than 2%.

Table 2. Accuracy study for analysis of KR and DQA

Analyte	Nominal concentration (µg/mL)	Found concentration* (µg/mL)	% Accuracy	%RSD
Kinetin Riboside	50	49.66 (±0.30)	99.31 (±0.60)	0.60
	100	101.32 (±0.26)	101.32 (±0.26)	0.26
	150	148.47 (±0.18)	98.98 (±0.12)	0.12
Dequalinium chloride	50	50.37 (±0.77)	100.74 (±1.54)	1.53
	100	100.47 (±0.17)	100.47 (±0.17)	0.17
	150	147.04 (±1.48)	98.03 (±1.21)	1.00

*Data expressed as mean (±SD); n=3.

Precision

The method showed acceptable precision study and % RSD was found to be less than 2% which is acceptable for precision study outlined by ICH guidelines (**Table 3**).

Table 3. (a) Precision study of KR (Day 1); (b) Precision study of DQA (Day 1); (c) Precision study of KR (Day 2); (d) Precision study of DQA (Day 2)

(a)

Sr.No.	Retention time (min)	Peak area
1	5.58	3172.76
2	5.58	3184.08
3	5.58	3190.96
4	5.59	3196.83
5	5.59	3223.00
6	5.58	3207.80
Mean	**5.58**	**3195.90**
SD	**0.003**	**17.76**
%RSD	**0.05**	**0.55**

(b)

Sr.No.	Retention time (min)	Peak area
1	6.99	1564.03
2	6.99	1568.09
3	6.99	1571.90
4	6.99	1573.20
5	6.99	1585.01
6	6.99	1580.58
Mean	**6.99**	**1573.80**
SD	**0.001**	**7.79**
%RSD	**0.01**	**0.49**

(c)

Sr.No.	Retention time (min)	Peak area
1	5.58	3157.97
2	5.58	3166.30
3	5.58	3184.40
4	5.58	3177.22
5	5.58	3182.07
6	5.58	3170.63
Mean	5.58	**3173.10**
SD	**0.001**	**10.06**
%RSD	**0.01**	**0.32**

(d)

Sr.No.	Retention time (min)	Peak area
1	6.99	1579.98
2	6.99	1569.20
3	6.99	1572.52
4	6.99	1568.13
5	6.99	1568.23
6	6.99	1562.64
Mean	**6.99**	**1570.12**
SD	**0.001**	**5.79**
%RSD	**0.01**	**0.37**

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

The LOD and LOQ were calculated by the Signal to Noise ratio (S/N) method. The limit of detection for KR and DQA was found to be 3.26 µg/mL and 3.03 µg/mL, respectively as it showed S/N ratio of 3.3:1 when compared to baseline. The limit of quantitation for KR and DQA was found to be 10.48 µg/mL and 10.12 µg/mL, respectively to get S/N ratio of 10:1 compared to baseline.

Robustness

The robustness was performed by doing small but deliberate changes in terms of flow rate (±0.2 mL/min), the temperature of column (±5°C), wavelength of detection (±2 nm), and change in mobile phase composition (±2% organic phase change). The results of the robustness study are tabulated in **Table 4**.

Table 4. Robustness study

Sr. No.	Parameter	Analyte	Peak area*	Retention time* (Rt)	Tailing factor* (T)
1. **Change in flow rate**					
1.	0.8 mL/min	Kinetin Riboside	4013.75	6.58	1.45
		Dequalinium chloride	1969.09	7.92	1.56
2.	1 mL/min	Kinetin Riboside	3173.10	5.58	1.39
		Dequalinium chloride	1570.12	6.99	1.49
3.	1.2 mL/min	Kinetin Riboside	2690.18	5.05	1.39
		Dequalinium chloride	1336.39	6.57	1.49
2. **Change in column temperature**					
1.	25˚C	Kinetin Riboside	3345.56	6.99	1.39
		Dequalinium chloride	1573.9	7.00	1.50
2.	30˚C	Kinetin Riboside	3173.10	5.58	1.39
		Dequalinium chloride	1570.12	6.99	1.49
3.	35˚C	Kinetin Riboside	2846.77	5.60	1.39
		Dequalinium chloride	1522.90	7.03	1.59
3. **Change in wavelength**					
1.	267 nm	Kinetin Riboside	2846.77	5.60	1.39
		Dequalinium chloride	1385.63	7.03	1.59
2.	269 nm	Kinetin Riboside	3173.10	5.58	1.39
		Dequalinium chloride	1570.12	6.99	1.49
		Kinetin Riboside	2861.72	5.60	1.39

3.	271 nm				
		Dequalinium chloride	1367.78	7.01	1.58
4. **Change in mobile phase (Buffer: Methanol) composition**					
1.	78:22	Kinetin Riboside	2859.29	4.87	1.56
		Dequalinium chloride	1454.29	6.77	2.00
2.	80:20	Kinetin Riboside	3173.10	5.58	1.39
		Dequalinium chloride	1570.12	6.99	1.49
3.	82:18	Kinetin Riboside	2858.05	4.91	1.64
		Dequalinium chloride	1432.04	6.84	2.19

*Data expressed as mean; n=3

Forced degradation study

The forced degradation study was carried out under stress conditions including acidic, basic, neutral, oxidative, thermal, and photolytic conditions. The results showed that KR in its formulation showed one major degradation product (37% degradation) under acidic conditions, and % recovery was found to be 96.6%, whereas under basic conditions, 9% degradation was observed. Under photolytic conditions (UV light; 27h), 4 degradants were observed, amounting to around 59% degradation, and % recovery was determined to be 70%. No degradation products were observed under peroxide oxidation, neutral and thermal conditions. KR when subjected to degradation, underwent oxidative degradation (2.26% degradation), thermal degradation (3.33% degradation) and hydrolytic degradation (8% degradation), while the rest of the trend was similar to that observed with KR in the LR-loaded formulation. In the case of DQA, no degradants were observed either in the placebo or in KR-loaded formulations in any of the conditions except for basic and photolytic degradation. The detailed analysis of KR and DQA in the blank formulation and KR-loaded formulation under varied stress conditions is tabulated in **Table 5**. The overlay of chromatograms of blank, KR, blank formulation (placebo), and KR-loaded formulation in each of the stressed conditions are depicted in **Supplementary Fig. S2 (a-f). Fig. S2 (i)** represents chromatograms at 269 nm (Detection wavelength of KR), while **Fig. S2 (ii)** represents chromatograms at 310 nm (Detection wavelength of DQA).

Table 5. (a) Force degradation study of KR in formulation; (b) Force degradation study of KR (c) Force degradation study of DQA in placebo formulation (d) Force degradation study of DQA in formulation

(a)

Stress conditions	Treatment	% Assay	% Degradation	% Recovery
Unstressed	–	**96.36**	**–**	**96.36**
Acid	1 N HCl (60˚C, 48h)	56.20	36.95	96.67
Base	1 N NaOH (60˚C, 48h)	51.89	9.04	63.23
Peroxide Oxidation	Hydrogen peroxide (30%, RT, 7 days)	96.14	–	99.77
Temperature	Thermal (80˚C, 48h)	82.16	–	85.26
Hydrolysis	Water (RT, 24h)	92.85	–	96.36
Photolysis	UV light (27h)	8.18	59.26	69.99

(b)

Stress conditions	Treatment	% Assay	% Degradation	% Recovery
Unstressed	–	**96.36**	**–**	**96.36**
Acid	1 N HCl (60˚C, 48h)	87.08	5.22	95.79
Base	1 N NaOH (60˚C, 48h)	75.75	7.56	86.45
Peroxide Oxidation	Hydrogen peroxide (30%, RT, 7 days)	80.26	2.26	85.63
Temperature	Thermal (80˚C, 48h)	92.98	3.33	99.91
Hydrolysis	Water (RT, 24h)	87.43	7.99	99.03
Photolysis	UV light (27h)	54.54	33.31	91.17

(c)

Stress conditions	Treatment	% Assay	% Degradation	% Recovery
Unstressed	–		–	**99.62**
Acid	1 N HCl (60˚C, 48h)	92.89	–	93.24
Base	1 N NaOH (60˚C, 48h)	9.82	72.35	82.48
Peroxide Oxidation	Hydrogen peroxide (30%, RT, 7 days)	94.59	–	94.94
Temperature	Thermal (80˚C, 48h)	92.21	–	92.56
Hydrolysis	Water (RT, 24h)	83.51	–	83.83
Photolysis	UV light (27h)	89.41	–	89.75

(d)

Stress conditions	Treatment	% Assay	% Degradation	% Recovery
Unstressed	–	**99.62**	–	**99.62**
Acid	1 N HCl (60˚C, 48h)	92.89	–	93.24
Base	1 N NaOH (60˚C, 48h)	11.81	80.66	92.82
Peroxide Oxidation	Hydrogen peroxide (30%, RT, 7 days)	94.59	–	94.94
Temperature	Thermal (80˚C, 48h)	92.21	–	92.56
Hydrolysis	Water (RT, 24h)	83.51	–	83.83
Photolysis	UV light (27h)	16.59	82.79	99.76

Applications of validated RP-HPLC method

Formulation and characterization of KR-loaded DQAsomes

Initially, preliminary batches of DQAsomes were formulated to assess the impact of any formulations and process parameters on particle size and zeta potential. Based on preliminary investigation, it was found that probe sonication amplitude had the highest impact on particle size and zeta potential. Thus, 3 batches, namely, F1, F2, and F3, were formulated and subjected to different probe sonication amplitudes, viz., 30, 50, and 70A, respectively. The formulation and process parameters for 3 batches and their respective size of particles, polydispersity index and zeta potential were enlisted in **Table 6**. Out of three formulations, the F3 batch with a sonication amplitude of 70A had a particle size of 215.3±3.54 nm and a PDI 0.12±0.004. It can be seen that as probe sonication amplitude increased, particle size decreased

along with PDI. The plot of particle size and PDI is shown in **Fig. 4.** The formulation F3 showed a zeta potential of +39.4 ±3.04 mV (**Fig. 4)**.

Evaluation of % entrapment efficiency and drug loading of KR in formulated DQAsomes

The RP-HPLC method that has been validated, was used for the evaluation of entrapment efficiency and drug loading of KR in DQAsomes by the equations (**Eqs. 4 and 5**) stated earlier. The % entrapment efficiency of the formulation with a probe sonication amplitude of 70A was found to be 81.12±5.62% and %drug loading was found to be 45.23±4.83% (**Table 6**).

Table 6. Formulation and process parameters and Characterization of KR-loaded DQAsomes

Parameters	**Formulations**		
	F1	**F2**	**F3**
KR conc (mM)	15	15	15
DQA conc (mM)	15	15	15
Rotational speed (rpm)	100	100	100
Rotational temperature (˚C)	35	35	35
Methanol volume (mL)	10	10	10
HEPES volume(mL)	10	10	10
Probe sonication time (h)	1	1	1
Probe sonication amplitude (A)	30	50	70
Particle size (nm)	**344.97±6.11**	**289.50±9.41**	**215.32±3.54**
PDI	**0.26±0.03**	**0.20±0.01**	**0.12±0.004**
Zeta potential (mV)	**24.47±1.50**	**29.2±4.20**	**39.4±3.04**
% Entrapment efficiency	**71.17±6.78**	**75.90±7.12**	**81.12±5.62**
% loading capacity	**35.30±3.61**	**43.29±7.54**	**45.23±4.83**

In vitro drug release study of KR-loaded DQAsomes

In vitro drug release from DQAsomes was performed by dialysis bag membrane (MWCO 14 kDa) for 3 batches, namely, F1, F2 and F3 [31]. F3 showed initial burst release to a greater extent (25%) than rest 2 formulations in 15 minutes. This might be attributed to the release of unentrapped KR from DQAsomes. After 1 h, the F3 batch showed 38.6% release while F1 and F2 showed 21% and 36.6% cumulative KR release, respectively. F2 and F3 batch showed 40% and 45% cumulative KR release, respectively, till 24 h, which confirmed the sustained release of KR from DQAsomes. The overall *in vitro* drug release profile can be well correlated with particle size obtained for 3 batches, with F1 having a greater particle size and

showing less drug release than F3, having a smaller particle size with the highest drug release. An *In vitro* cumulative drug release profile for 3 optimization batches of DQAsomes is depicted in **Fig. 5.**

Conclusion

The RP-HPLC method for the quantitation of KR in the formulation was found to be robust, accurate, and precise. The developed method has a short run time with good resolution between the two peaks of analytes. The method was further validated by ICH guidelines Q2(R1), which determined that the percent RSD for all enlisted chromatographic parameters was well within the ICH-specified limits. The linearity was established from 10 µg/mL to 250 µg/mL for both analytes, KR and DQA, with r^2 value of > 0.999. Through forced degradation studies, it was concluded that the drug degraded only under extreme conditions of acid, base, and UV light. Furthermore, KR-loaded DQAsomes were formulated and their particle size and zeta potential were characterized. The validated method can be utilized for the quantification of KR from DQAsomes with the determination of % entrapment efficiency and % drug loading along with *in vitro* drug release.

Declarations

Disclosure statement: None

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Author contributions: Formulation development, Sample analysis, Writing an original draft, Editing, Visualization, and Compilation of data were performed by Tejas Girish Agnihotri. Devendra Badgujar performed Formal data analysis, Validation, and Data collation. Dr. Nitish Sharma was involved in Conceptualization, Supervision, Data interpretation, Reviewing and editing the manuscript. Investigation, curation, reviewing and editing the manuscript were performed by Dr. Aakanchha Jain. All authors reviewed the manuscript.

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Figures

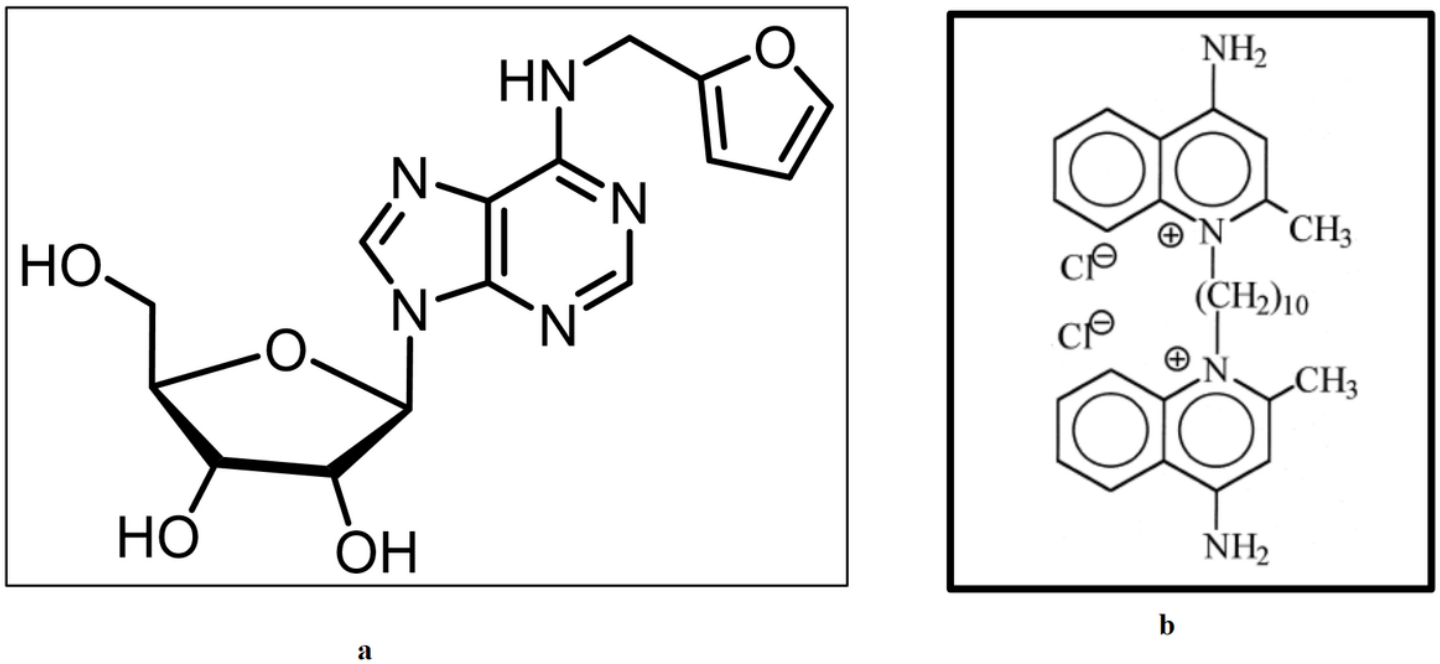

Figure 1

(A) Chemical structure of kinetin riboside

(B) Chemical structure of kinetin riboside

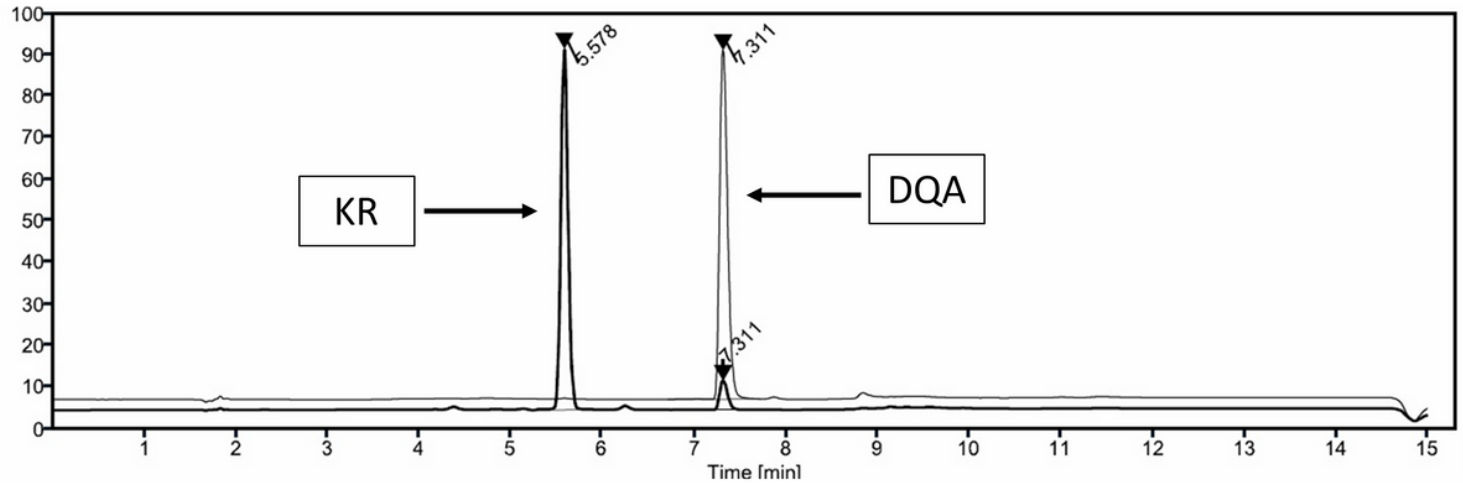

Figure 2

HPLC chromatogram of Kinetin Riboside and Dequalinium chloride

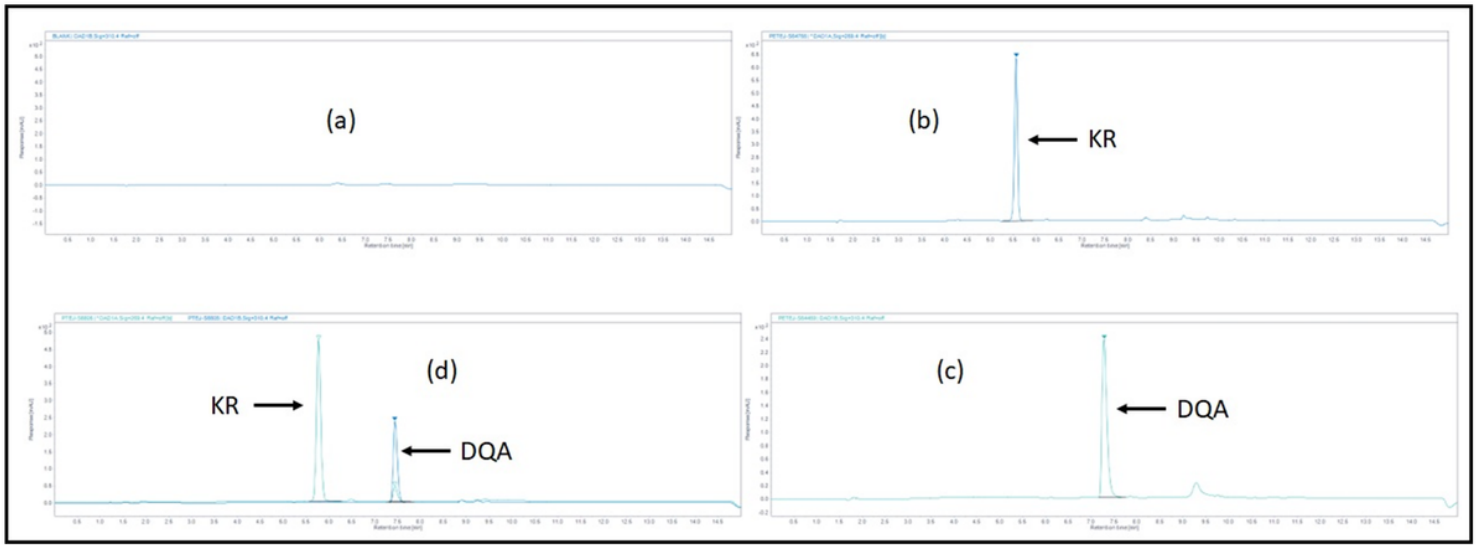

Figure 3

Chromatograms of (a) Blank-Methanol (b) Kinetin Riboside (c) Placebo formulation (without Kinetin riboside) (d) Kinetin Riboside loaded DQAsomes

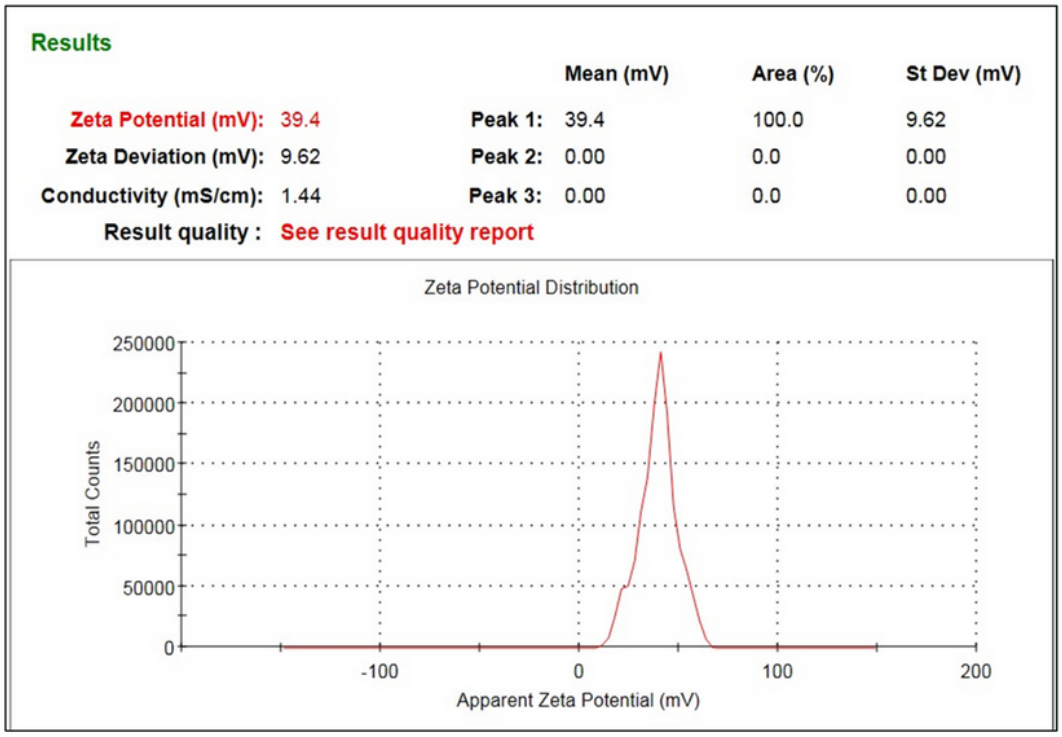

Figure 4

Plot of particle size, size distribution and zeta potential of KR-loaded DQAsomes

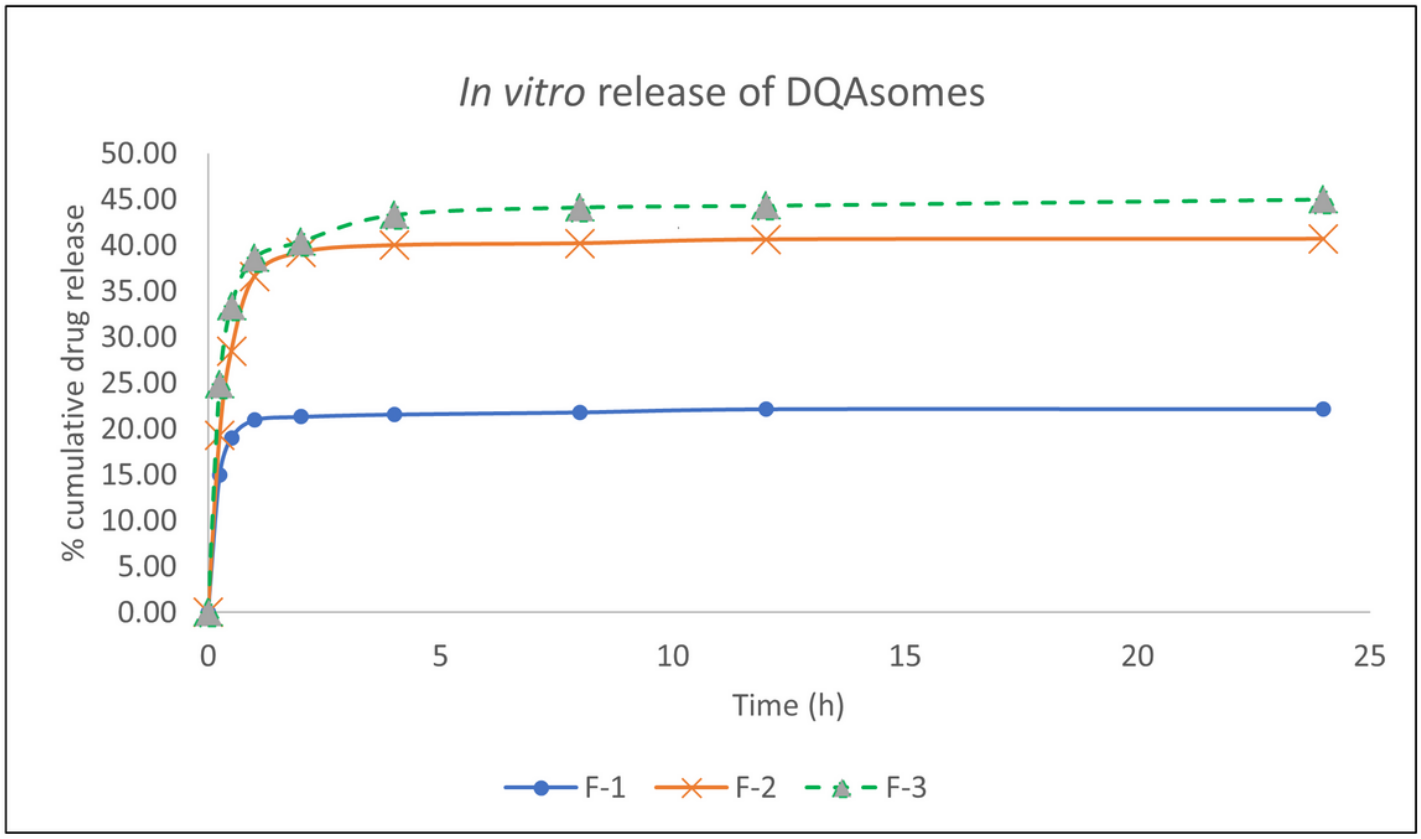

Figure 5

In vitro cumulative KR release profile of 3 batches of KR-loaded DQAsomes

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