

Cite this: *Anal. Methods*, 2024, 16, 3847Received 3rd May 2024  
Accepted 1st June 2024

DOI: 10.1039/d4ay00814f

rsc.li/methods

# Determination of 3-acetyl-11-keto- $\beta$ -boswellic acid in analytical and biological samples using streamlined and robust RP-HPLC method†

Sakshi Priya and Gautam Singhvi \*

AKBA (3-acetyl-11-keto- $\beta$ -boswellic acid) is a phytoconstituent derived from *Boswellia serrata* extract and utilized in the management of rheumatoid arthritis. Drug delivery approaches showed interest in delivering AKBA with advanced nanotechnology. There is a need for a simple, sensitive, and robust HPLC method that can determine AKBA in complex nanoformulation and *in vitro* and *ex vivo* samples. In the proposed work, the RP-HPLC method was developed using a mobile phase comprising a mixture of acetonitrile : milli Q (90 : 10) at detection  $\lambda_{\text{max}}$  250 nm. The method exhibited a linearity of 250 to 20 000 ng mL<sup>-1</sup> with a high correlation coefficient of 1. The limit of detection and limit of quantification for the analytes were found to be 41.32 ng mL<sup>-1</sup> and 125.21 ng mL<sup>-1</sup>, respectively. In the accuracy study, the % recovery of AKBA was found to be 98% to 102%, and the precision study showed less than 2% relative standard deviation. The developed method was found to be robust under chromatographic conditions with changes in pH and mobile phase mixture ratio. The method was also explored for forced degradation study, and the results showed the successful separation of degradation products from the AKBA. Further, the RP-HPLC method was applied for the quantification of AKBA in topical nanoformulations and different matrices, such as skin matrices and adhesive tapes. The method was able to measure entrapment efficiency ( $93.13 \pm 1.94\%$ ), drug loading ( $25.83 \pm 0.54\%$ ), drug assay in a gel matrix ( $96.99 \pm 3.89\%$ ), drug amount in stratum corneum ( $7.90 \pm 0.62 \mu\text{g cm}^{-2}$ ), and drug amount in viable skin layers ( $33.94 \pm 0.21 \mu\text{g cm}^{-2}$ ) with high-speed reproducibility. The developed method can be utilized for the routine analysis of AKBA in conventional and complex formulations in academia and industry.

## 1. Introduction

3-Acetyl-11-keto- $\beta$ -boswellic acid (AKBA) is a naturally derived pentacyclic triterpene obtained from the gum resin of *Boswellia serrata*. Its molecular weight is around 512.7 g mol<sup>-1</sup> with an empirical formula of C<sub>32</sub>H<sub>48</sub>O<sub>5</sub>.<sup>1</sup> AKBA exists as a white crystalline powder with limited water solubility and high solubility in commonly used organic solvents such as ethanol, methanol, acetonitrile, chloroform, and acetone.<sup>2,3</sup> Recently, it has become a drug of choice for the treatment of inflammatory conditions like rheumatoid arthritis (RA)<sup>4</sup> because of its immunomodulatory, anti-inflammatory, and 5-lipoxygenase (5-LOX) inhibitory properties.<sup>5–7</sup> AKBA is, therefore, able to reduce the tissue damage brought on by chronic inflammation by inhibiting the synthesis of inflammatory mediators through such mechanisms. Due to the possible health advantages of AKBA, it has

generated proficient attention in the realm of pharmaceutical research.<sup>3</sup>

AKBA is explored for oral and topical delivery with multifaceted nanocarrier systems for its effective delivery in RA.<sup>8</sup> Further, to increase the availability of AKBA at the disease site, the topical nanoformulations of AKBA can be a potential drug delivery approach. Developing and optimizing such AKBA-loaded nanocarrier-based delivery systems require a rapid and sensitive analytical method to evaluate *in vitro*, *ex vivo*, and *in vivo* samples. A sensitive method is required that can determine AKBA in the presence of various sample matrices, including solvents, buffers, and skin tissues.<sup>9,10</sup>

Analysis of AKBA using high-performance thin-layer chromatography, liquid chromatography-mass spectrometry, and high-performance liquid chromatography coupled with tandem mass spectrometry has been majorly reported for the quantification of different derivatives of boswellic acids such as AKBA, 11-keto-beta-boswellic (KBA), *etc.* or in combination with another analyte, in bulk drugs, dogs supplements and biological fluids such as rat plasma.<sup>11–14</sup> Miscioscia *et al.* have developed a high-performance liquid chromatography (HPLC) method for the quantification of AKBA and KBA in *Boswellia serrata* dog supplements. In this method, the optimized

Industrial Research Laboratory, Department of Pharmacy, Birla Institute of Technology and Science (BITS) Pilani, Pilani Campus, Rajasthan 333031, India. E-mail: gautam.singhvi@pilani.bits-pilani.ac.in

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4ay00814f>



## 2.4 Preparation of stock, calibration, and quality control standard solutions

A primary stock solution of 1 mg mL<sup>-1</sup> concentration was prepared with 10 mg of AKBA dissolved in 10 mL ACN. The secondary stock solution (100 µg mL<sup>-1</sup>) was prepared in ACN from the primary stock solution. The calibration standard solutions (250–20 000 ng mL<sup>-1</sup>) were prepared through serial dilution using ACN from a secondary stock solution.<sup>20</sup> Similarly, the three different quality control (QC) standard solutions, which are Low QC (LQC, 500 ng mL<sup>-1</sup>), medium QC (MQC, 2500 ng mL<sup>-1</sup>), and high QC (HQC, 15 000 ng mL<sup>-1</sup>), were prepared from the secondary stock solution.

## 2.5 Determination of validation parameters of the developed RP-HPLC method

The RP-HPLC method was developed and fully validated as per ICH Q2 (R1) guidelines for system suitability, linearity, range, LOD, LOQ, accuracy, intraday & interday precision, specificity, carryover effect, robustness, and solution stability.<sup>21</sup>

**2.5.1 System suitability.** For chromatographic methods, a system suitability test was recommended to guarantee that the system is effective and produces repeatable results. The system's efficacy was assessed by injecting the same sample with a concentration of 5 µg mL<sup>-1</sup> six times under optimal chromatographic conditions. The chromatogram was used to evaluate the peak area, retention time, theoretical plate, and tailing factor and considered acceptable when a theoretical plate number is more than 2000 and a tailing factor of <2.<sup>22</sup>

**2.5.2 Linearity, range, limit of detection, and limit of quantification.** The linearity of the chromatographic method was determined by injecting six standard concentrations of AKBA in the range of 250–20 000 ng mL<sup>-1</sup> using HPLC at 250 nm.<sup>23</sup> After acquiring the results, these were incorporated into the linear regression equation. Subsequently, the graph of the calibration curve was constructed between the analyte peak area on the y-axis and analyte concentrations on the x-axis. The obtained linear regression equation was assessed for the slope, regression coefficient, and intercept. The signal-to-noise (S/N) ratio was used to determine the LOD and LOQ. Initially, the detector response was used to determine the S/N ratio in the system suitability test. For LOD and LOQ, the ideal S/N ratios were 3 : 1 and 10 : 1, respectively.

**2.5.3 Accuracy and precision.** Accuracy is the determination of the closeness of an observed value to a specified value. Contrarily, precision is related to how closely measurement values are related to one another. Using the quality controls (low, medium, and high) and standard addition method, the accuracy was determined. Therefore, three different quality control level samples were determined using the calibration curve range, *i.e.*, LQC (500 ng mL<sup>-1</sup>), MQC (2500 ng mL<sup>-1</sup>), and HQC (15 000 ng mL<sup>-1</sup>), and were prepared from the standard stock solution. And for the standard addition method, a known quantity of standard stock solution was added to 80%, 100%, and 120% of the preanalyzed solution (10 000 ng mL<sup>-1</sup>). Each quality control level samples were prepared in *n* = 3. The %

recovery was used to indicate accuracy as per the ICH Q2 (R1) guideline.

The intraday precision was assessed at a different time point on the same day using the same prepared quality control level (LQC, MQC, and HQC) samples. Additionally, interday precision was assessed on different days (three consecutive days) using the same prepared quality control level (LQC, MQC, and HQC) samples. The % RSD was used to indicate precision as per the ICH Q2 (R1) guideline. The regulatory standards required that the precision and accuracy of QC samples be within <±2% RSD for approval.<sup>24</sup>

**2.5.4 Carryover effect.** Analyzing subsequent samples (250 ng mL<sup>-1</sup>, 2000 ng mL<sup>-1</sup>, and 10 000 ng mL<sup>-1</sup>) of the linearity curve after a blank allowed for the assessment of carryover.<sup>25</sup>

**2.5.5 Robustness.** The ICH standards define robustness as the analytical procedure's capacity to remain unaffected by minor modifications but intentional modifications in the experimental conditions. Various chromatographic parameters, including the mobile phase combination, column oven temperature, and pH, were chosen for the proposed experiments. Slight modifications to the operating parameters, such as the temperature of the column oven (±2 °C), mobile phase mixture (±2%), and pH of the milli Q (±0.5) were done.<sup>22,23</sup>

**2.5.6 Specificity study.** To evaluate the specificity of the developed chromatographic method, AKBA was identified amidst additional formulation excipients that are commonly found in nanoformulations and probably obstruct the drug's retention time. To ensure method specificity, the blank LNCs, and AKBA-loaded LNCs were centrifuged at 14 000 rpm for 30 min, followed by dilution of the supernatant with ACN. The prepared samples underwent filtration through a 0.22 µm syringe filter and were analyzed through HPLC.<sup>22,26</sup>

**2.5.7 Forced degradation studies of AKBA using the developed RP-HPLC chromatographic method.** As per ICH Q1A (R2) guideline, the stability-indicating nature of the developed chromatographic technique was analyzed under which the AKBA solution was subjected to different stress conditions such as acid hydrolysis, base hydrolysis, oxidation hydrolysis, and thermolytic hydrolysis. For acid and base hydrolysis, 1 M hydrochloric acid and 1 M sodium hydroxide were used to prepare a 200 µg mL<sup>-1</sup> solution of AKBA, respectively. The solution was then held for reaction on a water bath at 60 °C for 6 h. In a similar manner, AKBA solution was made with 3% hydrogen peroxide and heated for 6 h at 60 °C in a reflux environment to facilitate oxidative breakdown. For thermal stress, the AKBA solution was heated for 6 h at 60 °C in a reflux environment. All the stress conditions samples were also kept separately at room temperature to check the sample stability at room temperature. After keeping the AKBA solution (200 µg mL<sup>-1</sup>) under the aforementioned stress conditions, a 10 µg mL<sup>-1</sup> solution of AKBA was prepared using ACN. To safeguard the column, the acid and base samples were neutralized before being diluted with ACN. A 0.22 µm syringe filter was used to filter all the samples before they were injected into the HPLC for their analysis. The normal state and the chromatogram of various stress situations were compared. The different



degradant peaks and drug peaks were identified, and the percentage of AKBA degradation was determined.<sup>25</sup>

## 2.6 Application of validated RP-HPLC method

The developed and validated chromatographic method of AKBA was effectively utilized in analyzing stability-related samples of AKBA and quantifying AKBA in prepared LNCs. Furthermore, the method facilitated the analysis of AKBA entrapment efficiency in LNCs, *in vitro* release, *ex vivo* permeation, and skin retention samples. The application of this RP-HPLC chromatographic method is shown in Fig. 1.

**2.6.1 Solution stability study.** The developed method was utilized to assess the stability of the sample solution at varied temperature conditions such as fridge temperature (2–8 °C) and room temperature (25 ± 2 °C). For the same, the QC samples that were maintained at fridge temperature (2–8 °C) and room temperature, their accuracy and precision were examined on different days.<sup>27</sup>

**2.6.2 Preparation of AKBA-loaded lipid nanocarriers.** LNCs were formulated utilizing the hot emulsification process. The solid lipid (Dynasan 114) was melted first at 70 ± 5 °C, and then liquid lipid (Labrafac CC) was added along with AKBA to the melted lipid. The aqueous surfactant (Tween 80) phase was also heated at the same temperature. The lipid phase was progressively added dropwise to the heated aqueous phase while being stirred for 20 min. After hot stirring for 20 min, the prepared dispersion was sonicated for 6 min to reduce the size. Afterward, the dispersion was stirred for 30 min in an ice bath. The resulting formulation was allowed to cool at room temperature. Finally, the LNCs-loaded gel was obtained by loading AKBA-loaded LNCs dispersion into the gel matrix.<sup>28,29</sup>

**2.6.3 Entrapment efficiency and drug loading analysis.** For determination of entrapment efficiency and drug loading, the developed formulation underwent centrifugation at 5000 rpm for 5 min. Further, 100 µL supernatant was diluted with ACN to 1 mL and again kept for centrifugation for 30 min at 14 000 rpm. This step facilitated the setting of lysed nanoparticle content

and produced a clear supernatant. The supernatant was then diluted with ACN and filtered through a 0.22 µm syringe filter before injecting into the HPLC for their analysis.<sup>30,31</sup> The % entrapment efficiency and drug loading were calculated using the formula mentioned below.

$$\% \text{ Entrapment efficiency} = \frac{\text{amount of AKBA entrapped}}{\text{total amount of AKBA added}} \times 100$$

$$\text{Drug loading} = \frac{\text{weight of AKBA in LNCs}}{\text{weight of the other excipients and AKBA}} \times 100$$

**2.6.4 AKBA loaded LNCs gel assay.** The assay of the AKBA-loaded LNC gel was determined by dissolving the required quantity of gel in ACN. The resulting samples were filtered through a 0.22 µm syringe filter prior to injection into the HPLC for analysis.

**2.6.5 *In vitro* drug release analysis.** For the *in vitro* drug release investigation, a dialysis membrane (Hi media, MW: 12 to 14 kDa) was utilized. As release media, 15 mL of pH 7.4 PBS media were selected, and 2 mL of formulation was added to the dialysis bag. At release assembly, the bag was maintained at 350 rpm and 37 ± 2 °C. To maintain sink conditions, 1 mL aliquots were taken out at intervals of 1, 2, 4, 6, 8, 12, and 24 h and replaced with fresh release medium. After the collection of samples at different time intervals, they were passed through a 0.22 µm filter and then analyzed through a developed chromatographic technique. Three sets of experiments were conducted. The acquired data were used to create a graph with time (x-axis) and percentage cumulative medication release (y-axis).<sup>26</sup>

## 2.7 Application in *ex vivo* skin studies

**2.7.1 Extraction of AKBA from skin matrix.** The recovery percentage of AKBA from the animal skin was investigated before doing the *ex vivo* study. Goat ear skin obtained from an

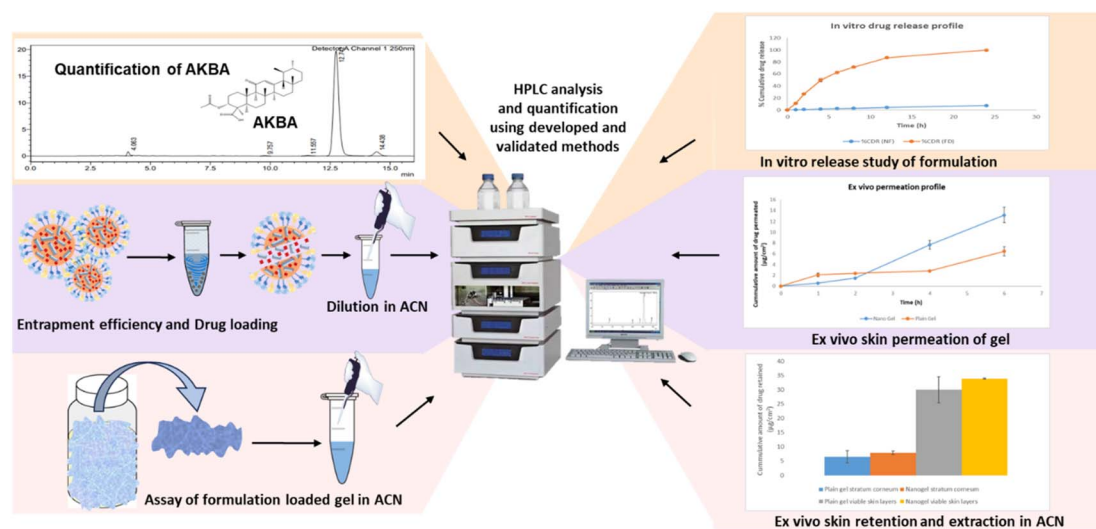


Fig. 1 Diagrammatic representation of the application of the developed RP-HPLC chromatographic method.



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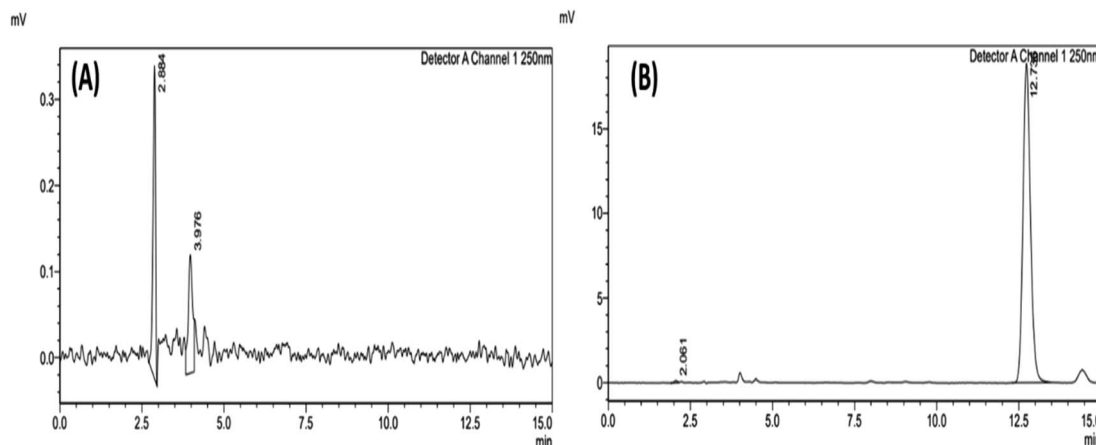


Fig. 2 (A) Chromatogram of blank ACN and (B) chromatogram of 10 000 ng mL<sup>-1</sup> AKBA sample.

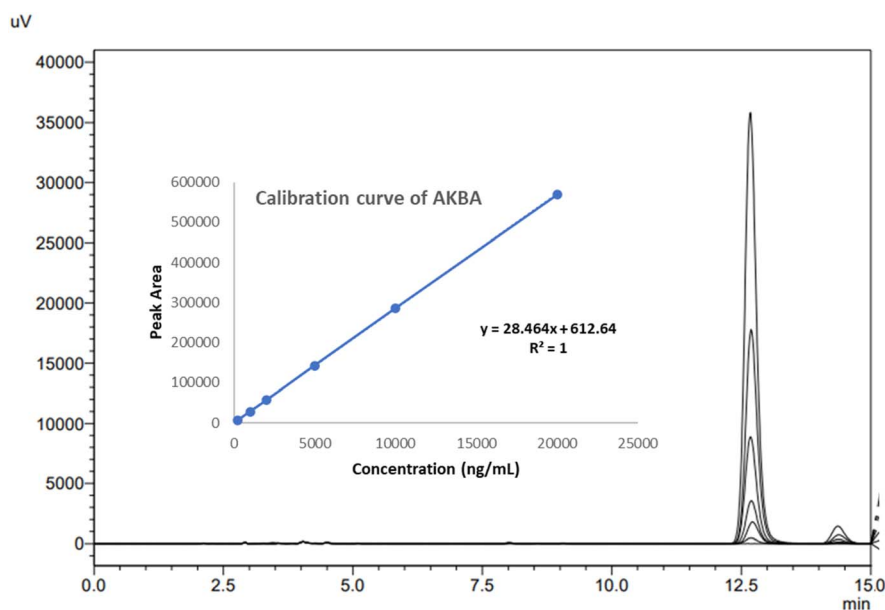
equation was found to be  $y = 28.464x + 612.64$ , where “x” represents the AKBA concentration while “y” is the peak area at 250 nm. Fig. 3 shows the details of the AKBA calibration curve. The signal-to-noise ratio was used to calculate the LOD and LOQ of the developed analytical technique. The results showed that the values were 41.3195 and 125.2108 ng mL<sup>-1</sup>, respectively. The outcomes demonstrated that the developed approach could identify and measure AKBA at nanogram levels with sufficient sensitivity. Routine analysis of AKBA in nanoformulation, where the drug is entrapped, might benefit from this technique.

**3.2.3 Accuracy and precision.** By using the quality controls (LQC (500 ng mL<sup>-1</sup>), MQC (2500 ng mL<sup>-1</sup>), and HQC (15 000 ng mL<sup>-1</sup>)) and the standard addition method, the accuracy was assessed. The method showed  $98.5039 \pm 0.8015$  to  $101.5737 \pm 1.2018\%$  recovery and % RSD less than 2%. The % recovery value (almost 100%) indicated accuracy in the measurement of the method without considerable variation. Further, the % RSD

value emphasized the consistency of the procedure across several runs and samples. Fig. 4 shows the overlay of chromatograms for LQC, MQC, and HQC. Table 1 provides the accuracy results in terms of % recovery and % RSD.

Further, by analyzing three quality control samples, *i.e.*, LQC (500 ng mL<sup>-1</sup>), MQC (2500 ng mL<sup>-1</sup>), and HQC (15 000 ng mL<sup>-1</sup>), the precision was analyzed. All the QC samples showed % RSD less than 2%, indicating the preciseness of the method. Table 2 provides the intra- and inter-day precision data in terms of % RSD for AKBA.

**3.2.4 Carryover effect.** The carryover impact was calculated by assessing subsequent samples with AKBA concentrations of 250, 2000, and 10 000 ng mL<sup>-1</sup>, followed by a blank sample. At 12.5 min into the retention period, there was no AKBA peak visible in the blank. The outcome showed that there was no carryover effect, meaning that this approach may be used continuously for a larger number of samples.



**Fig. 3** Calibration curve of AKBA along with an overlay of different calibration curve concentrations.

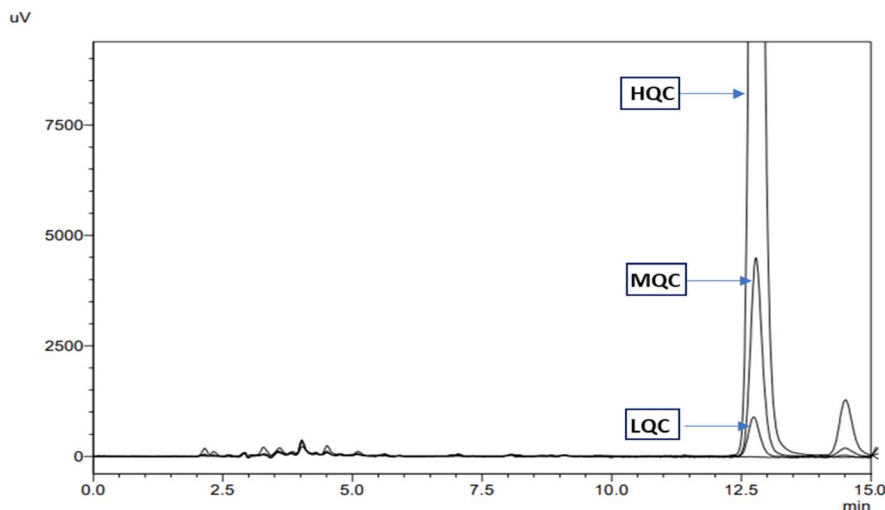


Fig. 4 Overlay of chromatograms for LQC, MQC, and HQC samples of AKBA.

Table 1 Accuracy determination of developed HPLC method for AKBA<sup>a</sup>

Using quality controls			Using standard addition method		
Concentration (ng mL <sup>-1</sup> )	% Recovery (mean $\pm$ SD) <i>n</i> = 3	% RSD	Amount added percentage (%)	% Recovery (mean $\pm$ SD) <i>n</i> = 3	% RSD
LQC (500)	98.5039 $\pm$ 0.8015	1.3874	80	101.3451 $\pm$ 0.7337	0.0072
MQC (2500)	99.3271 $\pm$ 0.6792	0.8052	100	100.3341 $\pm$ 0.7009	0.0069
HQC (15000)	101.5737 $\pm$ 1.2018	0.8256	120	100.3483 $\pm$ 0.9462	0.0094

<sup>a</sup> QC: quality control samples; LQC: lower quality control; MQC: medium quality control; HQC: high-quality control.

Table 2 Precision results of determination of AKBA using the HPLC method<sup>a</sup>

Sample QC	Intra-day repeatability RSD (%) ( <i>n</i> = 9)			Inter-day repeatability RSD (%) ( <i>n</i> = 9)		
	Morning	Afternoon	Evening	Day 1	Day 2	Day 3
LQC (500 ng mL <sup>-1</sup> )	0.5280	1.7693	1.8651	0.5280	1.3278	1.7037
MQC (2500 ng mL <sup>-1</sup> )	0.9437	1.0973	0.3747	0.9437	1.3943	1.2714
HQC (15 000 ng mL <sup>-1</sup> )	0.9674	0.8296	0.6798	0.9674	1.8432	1.7542

<sup>a</sup> QC: quality control samples; LQC: lower quality control; MQC: medium quality control; HQC: high-quality control.

**3.2.5 Robustness.** By altering the different chromatographic parameters, such as the pH of the milli Q (3 to 4), column oven temperature (28 to 32 °C), and mobile phase mixture ratio (88 : 12 to 92 : 08), robustness was evaluated. A significant difference was not observed in several essential

factors (peak area, tailing factor, and theoretical plate) by changing the above-mentioned parameters, as depicted in Table 3. By adjusting the variable circumstances, the developed method's robustness has been verified, and the observed values fall within the acceptable range. The analytical guidelines state

Table 3 Robustness results of the developed RP-HPLC method

Robustness parameters	Peak area mean $\pm$ SD (% RSD)	Tailing factor mean $\pm$ SD (% RSD)	HETP mean $\pm$ SD (% RSD)
Standard samples	146 132 $\pm$ 1542	1.16 $\pm$ 0.0015	9116 $\pm$ 77.5822
Column oven temp. (28 °C)	148 048 $\pm$ 6071 (0.9212%)	1.16 $\pm$ 0.0011 (1.7803%)	8889 $\pm$ 87.3059 (0.0405%)
Column oven temp. (32 °C)	147 874 $\pm$ 1446 (0.8377%)	1.16 $\pm$ 0.0045 (1.3293%)	9289 $\pm$ 91.0329 (0.0405%)
MP ratio (88 : 12)	143 817 $\pm$ 1113 (1.1292%)	1.15 $\pm$ 0.0030 (1.3152%)	8948 $\pm$ 78.6786 (0.9381%)
MP ratio (92 : 08)	146 471 $\pm$ 1556 (0.1636%)	1.19 $\pm$ 0.0045 (0.5178%)	9183 $\pm$ 104.9333 (1.4835%)
pH (3.0)	146 498 $\pm$ 1351 (0.1768%)	1.15 $\pm$ 0.0216 (0.0724%)	9106 $\pm$ 50.3421 (0.8151%)
pH (4)	145 380 $\pm$ 803 (0.3648%)	1.17 $\pm$ 0.0035 (0.8845%)	9002 $\pm$ 70.7695 (0.6654%)



that the theoretical plate number exceeding 2000, the tailing factor  $<2$ , and the % RSD  $\leq 2\%$  are acceptable. These results highlight the resilience of the system, guaranteeing consistent and dependable outcomes under various operating scenarios. It demonstrated that even in the event of slight variations in chromatographic conditions, the procedure can reliably produce accurate and precise results.

**3.2.6 Specificity study.** Lipids utilized in the preparation of LNCs were present during the specificity study. At the AKBA peak's retention time, no peak interference was detected, and the peak's 99.99% purity was determined. Fig. 5 illustrates the developed method's specificity, which clearly indicates the absence of a peak at the drug's RT. The method's ability to consistently and accurately measure AKBA without interference from the nanocarrier LNCs is confirmed by the unequivocal identification of the drug peak at its RT, without any comparable peak in the blank LNCs sample. These results strengthen the method's overall robustness and dependability and encourage its continued application in the development and quality control of nanocarrier systems loaded with AKBA.

**3.2.7 Forced degradation study of AKBA using the developed method.** The forced degradation studies in different stress conditions, including acid hydrolysis, base hydrolysis, oxidative hydrolysis, and thermolytic hydrolysis, were conducted successfully. Table 4 depicts the AKBA stability results. The AKBA sample's acid hydrolysis chromatogram (Fig. 6A)

displayed degradation peaks at different RT. Under acidic hydrolysis, 48.17% degradation of AKBA was seen. Similarly, the base hydrolysis chromatogram of the AKBA sample was shown in Fig. 6B, and 89.11% degradation of AKBA was observed. This suggests that AKBA can deteriorate in highly basic conditions if kept at a higher temperature for a longer duration. Additionally, the chromatogram of AKBA (Fig. 6C) was of oxidative stress conditions, and 5.15% degradation of AKBA was observed in oxidative stress conditions. The thermal hydrolysis chromatogram is shown in Fig. 6D, and 50.79% degradation of AKBA was observed in thermal hydrolysis conditions. The stress samples, which were kept at room temperature, were also analyzed, and it was observed that in all different conditions, the % recovery of AKBA was approximately more than 80 to 90% after 6 h. The outcomes demonstrated that the developed analytical approach was capable of differentiating between the degradation products and the AKBA. It was observed that the developed approach demonstrated the method's stability-indicating property and was selective for AKBA. This may be used for AKBA in-process and final product stability testing.

### 3.3 Application of developed and validated RP-HPLC chromatographic method

**3.2.5 Solution stability study.** The solution stability study was performed at two temperatures, room temperature ( $25 \pm 2^\circ\text{C}$ ) and fridge temperature ( $2$  to  $8^\circ\text{C}$ ), for 7 days. The samples,

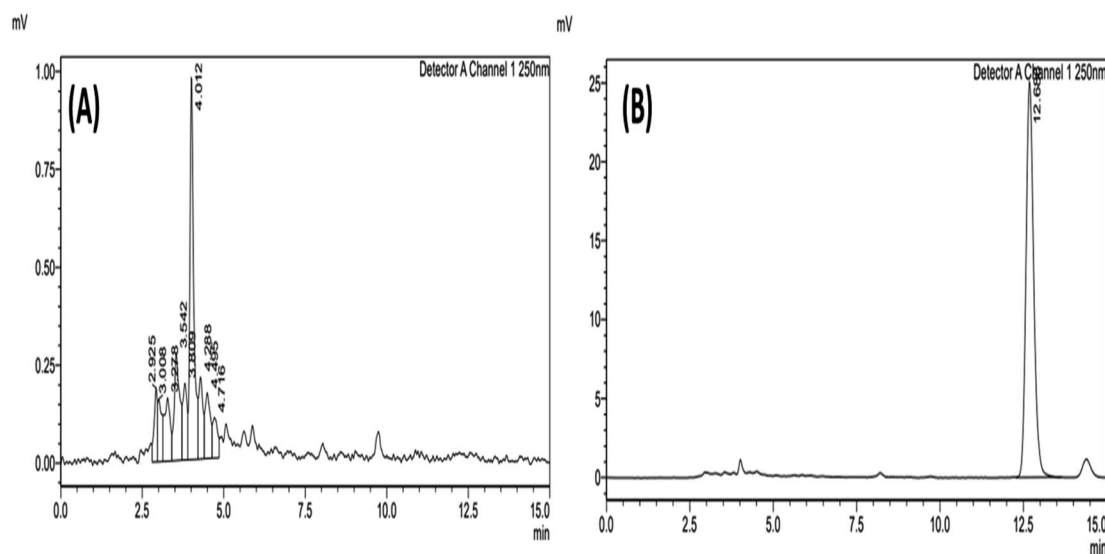


Fig. 5 (A) Chromatogram of blank LNCs and (B) chromatogram of AKBA loaded LNCs.

Table 4 Forced degradation results of AKBA in different stress conditions

Stress condition	Solvent	Temperature	Sampling time	% of AKBA degraded $\pm$ SD
Acid hydrolysis	1 N HCl	60 $^\circ\text{C}$	6 h	48.1777 $\pm$ 1.3060
Base hydrolysis	1 N NaOH	60 $^\circ\text{C}$	6 h	89.1178 $\pm$ 1.4982
Oxidative hydrolysis	30% $\text{H}_2\text{O}_2$	60 $^\circ\text{C}$	6 h	5.1576 $\pm$ 7.1665
Thermal hydrolysis	Water	60 $^\circ\text{C}$	6 h	50.7991 $\pm$ 14.3801



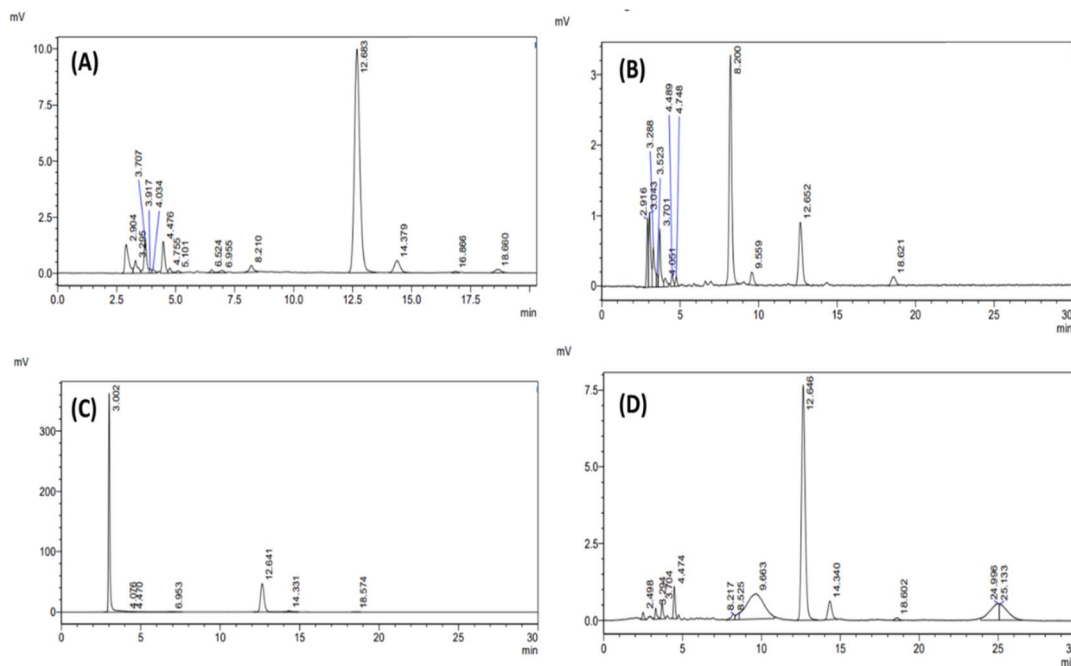


Fig. 6 RP-HPLC chromatogram of (A) acid hydrolysis, (B) base hydrolysis, (C) oxidative hydrolysis, and (D) thermolytic hydrolysis.

which were kept at room temperature and fridge temperature, were found to be stable till 3 days and 7 days, respectively. After 3 days of room temperature samples, the % RSD for peak area, RT, HETP, and TF was observed at 1.6810, 0.3576, 1.2498, and 0.4538, respectively. Similarly, after 7 days of fridge temperature samples, the % RSD for peak area, RT, HETP, and TF was observed at 1.7618, 0.9530, 1.2627, and 0.3973, respectively. The method's efficiency in monitoring AKBA stability is demonstrated by the quantitative values produced, which exhibit low variability and excellent accuracy. This application showed hope for the utilization of this method for several pre-formulation studies required for formulation development.

**3.3.2 Entrapment efficiency and drug loading analysis.** The entrapment efficiency (%) and drug loading (%) of AKBA-loaded LNCs were found to be  $93.1380 \pm 1.9394\%$  and  $25.8280 \pm 0.5378\%$ , respectively. The results of entrapment efficiency and drug loading showed that the developed method quantified the AKBA in AKBA-loaded LNCs with great precision and accuracy. This confirmed the proficiency of the developed RP-HPLC chromatographic method in measuring the AKBA in complex nanoformulations of AKBA.

**3.3.3 AKBA loaded LNCs gel assay analysis.** The assay of AKBA-loaded LNC gel was performed, and it was observed that  $96.9923 \pm 3.8924\%$  of AKBA was present in the AKBA-loaded LNC gel. The percentage recovery amount showed that the method was specific and sensitive in determining the active constituent in the presence of formulation excipients.

**3.3.4 *In vitro* drug release analysis.** The *in vitro* drug release study showed  $99.6718 \pm 0.9998\%$  release from free AKBA solution and  $7.2816 \pm 0.3497\%$  release from AKBA-loaded LNCs formulation. Furthermore, no interference was identified at drug RT because of dissolution media. Also, the method was

able to detect the lower amount of drug released ( $0.39 \mu\text{g mL}^{-1}$ ) from the AKBA-loaded LNCs at the initial time point. Fig. 7 shows the *in vitro* release profile of AKBA from free AKBA solution and AKBA-loaded LNCs.

### 3.4 Application in *ex vivo* skin studies

**3.4.1 Extraction of AKBA from skin matrix.** The percentage of AKBA recovered from skin tissue was  $86.7407 \pm 3.1736\%$ . Persistent recovery and utility on AKBA measurement in skin permeation and retention investigations were demonstrated by the developed RP-HPLC chromatographic method.

**3.4.2 AKBA skin permeation and retention study.** The skin permeation and retention study through goat ear skin was performed, and it was observed that  $6.4634 \pm 0.8333 \mu\text{g cm}^{-2}$  of AKBA was permeated from the plain gel and  $13.1823 \pm 1.4111$

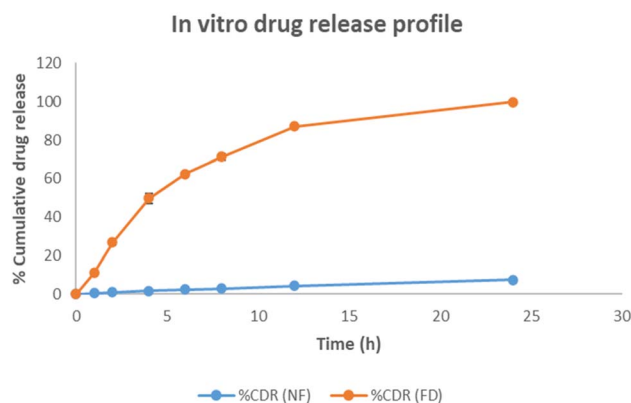


Fig. 7 *In vitro* release profile of AKBA from its complex nanoformulation.



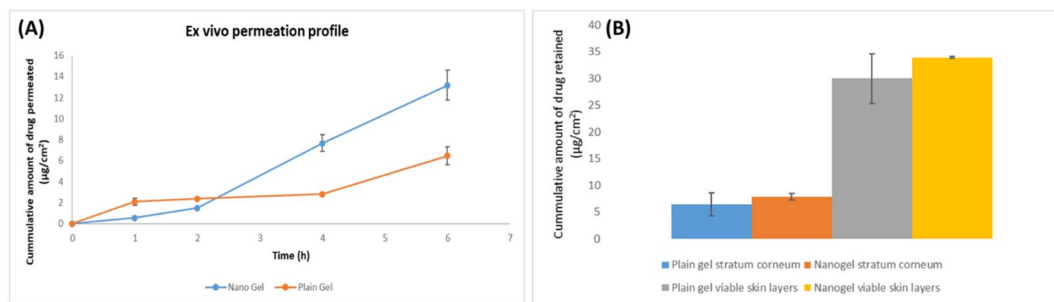


Fig. 8 (A) AKBA *ex vivo* skin permeation profile from AKBA loaded LNCs (data represented as mean  $\pm$  SD,  $n = 3$ ), (B) bar graph representing the amount of AKBA in stratum corneum and viable skin layers (data represented as mean  $\pm$  SD,  $n = 3$ ).

$\mu\text{g cm}^{-2}$  of AKBA was permeated from AKBA loaded LNCs gel in 6 h. The *ex vivo* skin penetration profile of AKBA-loaded LNCs is displayed in Fig. 8A. The drug quantity in the stratum corneum was determined to be  $6.4204 \pm 2.1762 \mu\text{g cm}^{-2}$  and  $7.9037 \pm 0.6174 \mu\text{g cm}^{-2}$  and  $30.0031 \pm 4.6639 \mu\text{g cm}^{-2}$  and  $33.9446 \pm 0.2006 \mu\text{g cm}^{-2}$  in viable skin layers from plain gel and AKBA loaded LNCs gel respectively. AKBA retention in skin layers is seen in Fig. 8B. It was observed that the developed method was found to be efficient in determining the drug content in *ex vivo* studies. The method showed utility for further exploration in dermatopharmacokinetic studies of AKBA.

## 4. Conclusion

The development of the HPLC method is crucial for pharmaceutical products, drug development, and drug discovery. In the present work, a novel, simple, sensitive, robust, reproducible, economic, and stability-indicating RP-HPLC method was developed. By monitoring the conditions that produced the optimum peak parameters, the composition of the mobile phase and the chromatographic conditions were optimized. Afterward, the method underwent thorough validation as per ICH Q2 (R1) guidelines, demonstrating to be linear in the concentration range, sensitive, accurate, precise, stable, and robust. Compared to the pre-existing method reported in the literature, the current method possesses several benefits, such as a detailed explanation of the optimization process of the developed methods, shorter retention time and run time, high sensitivity, eliminating variability in accuracy and precision, demonstrating significant robustness across a variety of conditions, and reflecting stability-indicating method for AKBA. Additionally, the current method is specific to the estimation of pure AKBA alone. The efficacy of the established HPLC method in analyzing the stability of AKBA under various stress conditions, including acidic hydrolysis, basic hydrolysis, oxidative hydrolysis, and thermal hydrolysis, has been proven by a forced degradation study. The developed HPLC method yielded a thorough stability indicating nature and demonstrated robustness and reliability in the detection of AKBA in the presence of degradants.

The developed method is novel for the estimation of AKBA in AKBA-based nanocarrier systems, indicating a requirement to quantify AKBA in emerging drug delivery systems, which

eventually advances nanomedicine and improves therapeutic effectiveness. The developed method is able to estimate the AKBA in the presence of nanocarrier excipients in an *in vitro* drug release medium, *ex vivo* tape-stripped, and skin-permeated samples of AKBA at a biological pH medium. The usefulness of the validated analytical approach was demonstrated in the assessment of AKBA in pharmaceutical nanoformulations and for dermal product evaluation. As a result, the established approach may be applied in academia and industry to characterize and assess the stability of AKBA-loaded nanoformulations in a cost-effective way.

## Data availability

The data supporting this article have been included as part of the ESI.†

## Author contributions

Sakshi Priya: conceptualization; methodology; investigation; validation; data curation; formal analysis; writing – original draft; writing – review & editing. Gautam Singhvi: conceptualization; project administration; resources; supervision; validation; visualization; writing – review & editing.

## Conflicts of interest

Authors declare no conflict of interest.

## Acknowledgements

Sakshi Priya gratefully acknowledged the PhD fellowship from DST-INSPIRE, Govt. of India [#IF210090].

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