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# Simultaneous Determination of Cinchocaine Hydrochloride and Betamethasone Valerate in Presence of Their Degradation Products

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## Abstract

Cinchocaine hydrochloride (CIN) and betamethasone valerate (BMV) are co-formulated in pharmaceutical formulations that could be used for local treatment of hemorrhoids. Both drugs are susceptible to hydrolytic degradation. Two sensitive and precise stability-indicating chromatographic methods were developed for the simultaneous determination of both active pharmaceutical ingredients. The developed methods were applied for quantitation of CIN and BMV in their pure forms, in presence of their corresponding degradation products and in their pharmaceutical formulation. The first method was a high performance liquid chromatographic (HPLC) one, separation and quantitation was achieved using a Waters Spheriosorb® 5 μm ODS2 $C_{18}$ analytical column and an isocratic mobile phase formed of acetonitrile–acetate buffer (pH 6.5 ± 0.1) in a ratio of (55:45, v/v). The mobile phase was pumped at a flow rate of 1.2 mL/min. UV-detection was done at 240 nm using photodiode array detector. The second method was based on thin layer chromatography (TLC) fractionation coupled with densitometric determination. Separation was done on high performance thin layer chromatography (HPTLC) silica gel 60$F_{254}$ plates using a developing system formed of chloroform–toluene–ethanol–acetic acid in a ratio of (4.5:4.5:1:1, by volume). The separated bands were scanned densitometrically at 240 nm. For the HPLC method, linearity was confirmed over concentration ranges of 4–300 and 4–350 μg/mL for CIN and BMV, respectively. For the HPTLC-densitometric method, the obtained ranges were 0.5–12 and 0.5–10 μg/band for CIN and BMV, respectively. The developed methods were optimized and validated according to the ICH guidelines. CIN acid degradation products were separated and identified by mass spectroscopy. The developed HPLC method was used to study the kinetics of acid and alkali degradation of the both drugs. The results obtained were statistically analyzed and compared with those obtained by applying the official methods for both drugs.

## Introduction

Cinchocaine hydrochloride (CIN) is 2-butoxy-*N*-[2-(diethylamino) ethyl]quinoline-4-carboxamide hydrochloride, Figure 1a. It is a local anesthetic agent (1). Betamethasone valerate (BMV) is chemically known as 9-fluoro-11β, 21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate, Figure 1b. It is a glucocorticoid anti-inflammatory drug (1). The combination of CIN and BMV, formulated as ointment, is used for local treatment of hemorrhoids. CIN is known to be labile for acid degradation, while it shows relative stability towards alkali, oxidative and thermal stress conditions (2–4). BMV is easily degraded by alkali, acid and thermal stress conditions, while it shows relative stability under oxidative degradation (5–8).

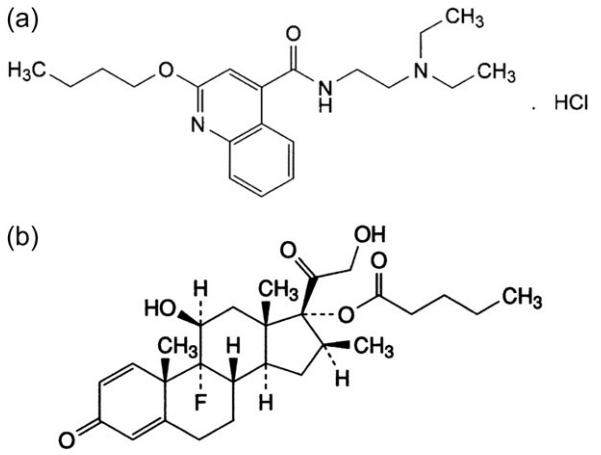


**Figure 1.** Chemical structures of cinchocaine hydrochloride (a) and betamethasone valerate (b).

As per the ICH guidelines (9), impurities in new drug products could be due to degradation products of the drug substances or side reactions with product excipients. Thus development of an analytical method for analysis of the active pharmaceutical ingredients in the presence of related impurities and degradation products is very important in the pharmaceutical industry.

There was no reported method for the simultaneous determination of both components either in their binary mixture or in the presence of their corresponding degradation products. The aim of this work was to develop and validate novel, simple, rapid and sensitive stability-indicating high performance liquid chromatographic (HPLC) and high performance thin layer chromatography (HPTLC)-densitometric methods for the determination of CIN and BMV in their combined pharmaceutical formulation and in the presence of their hydrolytic degradation products.

## Experimental

### Instrumentation

#### HPLC method

HPLC Agilent model 1260 infinity series was used. It consisted of a quaternary pneumatic pumping system (model G1311C) and a Rheodyne injector (model G1328C (equipped with 20-µL injector loop) (Agilent, Waldbronn, Germany). The detection was done using photodiode array detector (model G1315D, Agilent, Germany). Separation was done by Waters Spherisorb® ODS2 $C_{18}$ column, 4.6 × 250 mm, 5 µm particle size (Wexford, Ireland). A Soniclean 160T sonicator (Soniclean, Thebarton, Australia) was used for extraction of drugs from pharmaceutical formulations.

#### HPTLC-densitometric method

Silica gel 60$F_{254}$ pre-coated HPTLC aluminum sheets, 20 × 20 cm was obtained from Merck (Darmstadt, Germany). Camag Linomat five autosampler (Camag, Muttenz, Switzerland) with Camag micro syringe (100 µL) was used to apply the samples. Scanning was performed using a Camag thin layer chromatography (TLC) scanner model 3S/N 1302139 with winCATS software for densitometric evaluation (Camag, Muttenz, Switzerland). The measuring mode was reflectance, slit dimensions: 3 × 0.45 mm, scanning speed: 20 mm/s, output as a chromatogram and integrated peak area.

#### For MS (identification of CIN degradation products)

Samples were directly injected into the MS, triple quadruple MS 6420 Agilent equipped with mass hunter workstation software-data acquisition for 6,400 series (Santa Clara, CA, USA). The operating conditions were: positive mode ESI, gas temperature 275°C, gas flow 12 L/min, nebulizer gas pressure 60 psi and capillary voltage 3,000 V.

### Materials and Chemicals

#### Pure form

CIN standard was kindly supplied by Alexandria Co. Pharmaceuticals, Alexandria, Egypt. Its purity was assessed and found to be 100.05 ± 1.32% according to BP official method (1). BMV standard was kindly supplied by GlaxoSmithkline S.A.E., Cairo, Egypt. Its purity was checked and found to be 100.46 ± 1.17% according to BP official method (1). Betamethasone-21-valerate and betamethasone alcohol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany) with certified traceable purites of ≥95%.

#### Pharmaceutical formulation

Supraproct-S® ointment (Batch No. 0014) was manufactured by Julphar, Gulf Pharmaceutical Industries, Ras Al Khaimah, UAE. Each gram was labeled to contain 5 mg of CIN and 1 mg of BMV.

#### Chemicals

All chemicals used were of analytical grade, and the solvents were of HPLC grade. Sodium hydroxide, glacial acetic acid, toluene, ethanol (El Nasr Pharmaceutical Chemical Co., Egypt), triethylamine, chloroform, acetonitrile, *n*-hexane (Sigma-Aldrich, Steinheim, Germany), and anhydrous sodium acetate (E. Merck, Darmstadt, Germany). The water for HPLC was prepared by double distillation and filtration through a 0.22 µm nylon membrane filter (Agela Technologies, Wilmington, USA).

### Degraded samples

#### CIN acid induced degradation products preparation

Into a 100-mL rounded flask, a mass of 25 mg of CIN was transferred; 25 mL of 2M hydrochloric acid were then added and refluxed for 2 h. After reflux, the solution was cooled. The pH was then adjusted to 7 with a pre-calculated amount of 2M sodium hydroxide. Complete degradation was tested by the proposed chromatographic methods.

#### BMV alkali induced degradation products preparation

A mass of 25 mg of BMV was transferred into a small beaker and dissolved in 25 mL of acetonitrile. One milliliter of 2M sodium hydroxide solution was then added and the solution was allowed to stand for 90 min at room temperature, till complete degradation as tested by the proposed chromatographic methods. The pH was then adjusted to 7 with a pre-calculated amount of 2M hydrochloric acid.

### Stock solutions

#### Standard stock solutions of CIN and BMV (each, 1 mg/mL)

Standard stock solutions were prepared in a solvent mixture of acetonitrile: water (50:50, v/v) for the HPLC method and in methanol for HPTLC-densitometric method. All standard solutions were freshly prepared.

**Stock solution of CIN acid induced degradation products (0.2 mg/mL)**
Solution of CIN acid induced degradation products was prepared as under "Degraded samples" but using 20 mg of CIN; the volume was then completed to 100 mL with methanol.

**Stock solution of betamethasone alkali induced degradation products (0.2 mg/mL)**
BMV alkali induced degradation products solution was prepared as described above but using 20 mg of BMV and the volume was then completed to 100 mL with methanol.

**Laboratory prepared mixtures**
Different aliquots of CIN and BMV were accurately transferred into series of 10-mL volumetric flasks then calculated volumes of the degradation products solutions were added to prepare different mixtures. The volume was completed by solvent mixture of acetonitrile: water (50:50, v/v), for the HPLC method. While for HPTLC-densitometric method, the volume was completed by methanol.

## Chromatographic conditions

**For HPLC method**
The mobile phase was formed of acetonitrile and 25 mM acetate buffer containing 0.2% triethylamine (final pH was adjusted to 6.5 ± 0.1 using acetic acid), in a ratio of 55:45, v/v. The obtained mobile phase was filtered using 0.22 µm nylon membrane filter and degassed by ultra-sonication prior to use. Before injection, the samples were filtered using 0.22 µm disposable filters. The mobile phase was introduced isocratically at flow rate of 1.2 mL/min with UV–DAD detection at 240 nm. All determinations were performed at ambient temperature. The injecting sample volume was 20 µL. The analysis was usually performed after passing 20–30 mL mobile phase for conditioning of the stationary phase to reach equilibrium.

**For HPTLC-densitometric method**
Each drug standard solution was applied on 20 × 20 cm HPTLC aluminum sheets, pre-coated with silica gel $60F_{254}$, as separate compact bands 10 mm apart from each other and 10 mm from the sides and bottom edge of the plates with 6 mm band length, and 150 nL/s dosage speed. Linear ascending development was performed in a chromatographic tank pre-saturated with chloroform–toluene–ethanol– acetic acid (4.5:4.5:1:1, by volume) as a developing system for 30 min prior to use. The plates were developed over a distance of 8 cm then air dried at room temperature. The separated bands were scanned at 240 nm.

## Procedures

**Construction of calibration curves**
*For HPLC method.* Aliquots of standard solutions of CIN and BMV were, separately, transferred into a series of 10-mL volumetric flasks and diluted to volume with solvent mixture of acetonitrile: water (50:50, v/v) to yield solutions in the concentration range of 4–300 and 4–350 µg/mL, respectively. A volume of 20-µL of each solution was injected in triplicate and chromatographed under the previously mentioned chromatographic conditions. The average peak area obtained for each concentration was plotted versus the corresponding concentration to obtain the calibration curves then the regression equations were computed.

*For HPTLC-densitometric method.* Aliquots of standard solutions of the studied drugs equivalent to 0.5–14.0 mg of CIN and 0.5–10.0 mg of BMV were, separately, transferred into a series of 10-mL volumetric flasks; the volume was completed to the mark with methanol. Each solution of 10-µL were separately applied in triplicates as bands onto HPTLC plates. The chromatographic conditions were followed and the peak areas were recorded. The calibration curves were constructed and the regression equations were computed.

*Application to pharmaceutical formulation.* Into a 100-mL beaker, an amount of 2.0 g ointment was accurately weighed and dispersed into 20 mL *n*-hexane with aid of stirring and sonication for 15 min. The solution was quantitatively transferred into a 250-mL separating funnel. Shaking was done for 3 min till complete dispersion of the ointment. The solution was extracted three times, each with 15 mL of the solvent mixture (acetonitrile: water, 50:50, v/v). The lower layer, containing the active ingredients, was then transferred into a 50-mL volumetric flask. The volume was completed to mark with the same solvent mixture and filtered through filter paper to obtain concentrations of 200 µg/mL of CIN and 40 µg/mL of BMV.

For the HPTLC-densitometric method, a volume of 25 µL from the prepared solution was spotted. For the HPLC method, appropriate dilutions were carried out with solvent mixture (acetonitrile: water, 50:50, v/v) to obtain solutions having concentrations of 50 µg/mL of CIN and 10 µg/mL of BMV. The procedures mentioned under the two methods were followed. By applying the corresponding regression equations, the concentrations of CIN and BMV and then the mean recoveries were calculated.

*Identification of the acid induced degradation products of CIN.* For identification of CIN acid degradation products, the conditions mentioned under "Degraded samples" were applied.

*Kinetic studies.* To determine the rate of acid and alkali degradation of the drugs, kinetic studies were carried out for a drug concentration of 1 mg/mL under the previously mentioned stress conditions. Samples were withdrawn at 15 and 5-min intervals for CIN (stress acid degradation) and BMV (stress alkali degradation), respectively, then neutralized by adding appropriate solutions. Analysis was done using the developed HPLC method.

# Results

## CIN degradation study

CIN was reported to undergo degradation in acid medium. The proposed degradation pathway was suggested to set forth through the formation of *N*-[2-(diethylamino)ethyl]-2-hydroxyquinoline-4-carboxamide (Deg. I). Further degradation of (Deg. I) gives 2-hydroxyquinoline-4-carboxylic acid (Deg. II) (2, 3). El-Gindy *et al.* (4) separated one acid degradation product of CIN, Deg. I, as proved by spectral analysis. In this study, CIN was subjected to acid degradation for 2 h by refluxing with 2M hydrochloric acid. Complete degradation was achieved by disappearance of the HPLC peak and TLC spot corresponding to the intact drug. The products were subjected to MS to identify them. Figure 2 shows MS1 scan, where three degradation products were identified. The *m/z* values of these products confirm the postuated degradation pathway as shown in (Figure 3). The use of MS1 with ESI mode (soft ionization) allows the identification of molecular ions with minimum further fragmentation.

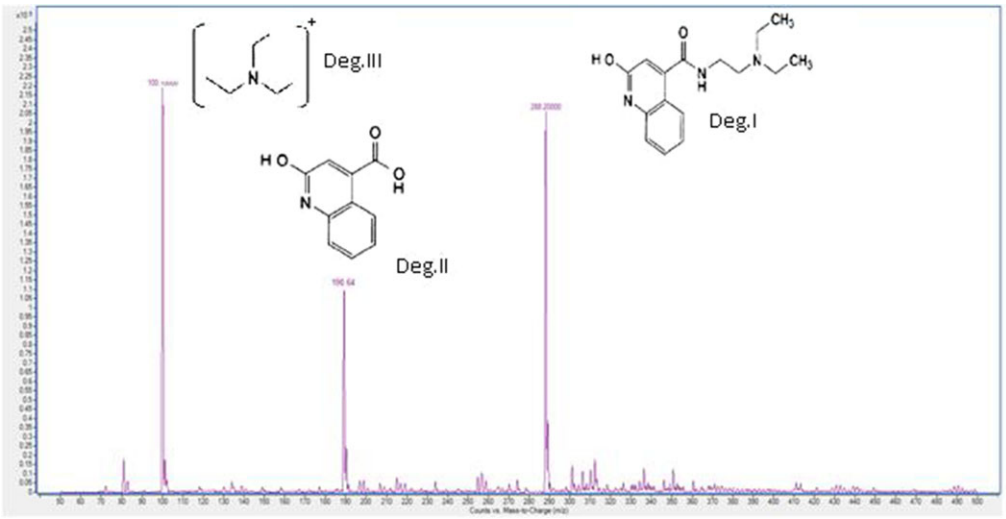


**Figure 2.** MS of cinchocaine hydrochloride acid induced degradation products.

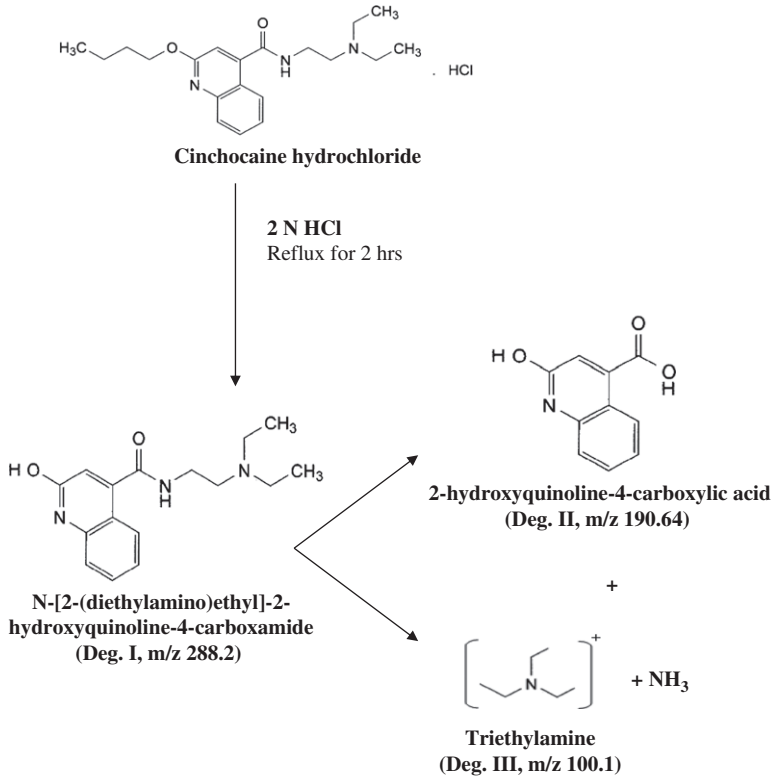


**Figure 3.** The suggested pathway for cinchocaine hydrochloride acid induced degradation.

## BMV degradation study

Betamethasone 17-valerate was reported to undergo rapid degradation through re-arrangement to the isomer betamethasone-21-valerate (BMV-21) in alkali medium, followed by catalyzed hydrolysis to betamethasone alcohol (BMV-alcohol) (5–7) as illustrated in Figure 4. It is also susceptible to acid catalyzed hydrolysis but at a rate much less than base catalyzed hydrolysis. The degradation products are generally less active than the parent compound. Betamethasone 21-valerate has one-15th the activity of 17-valerate. If conversion to the less active product takes place in the formulated products, the change could be of clinical significance (8). Both degradation products were stated as potential impurities in the BP (1). In this study, BMV was subjected to complete alkali degradation using 2M NaOH for 2 h at ambient temperature. Also, testing of BMV thermal degradation was performed by heating methanolic solution at 80°C.

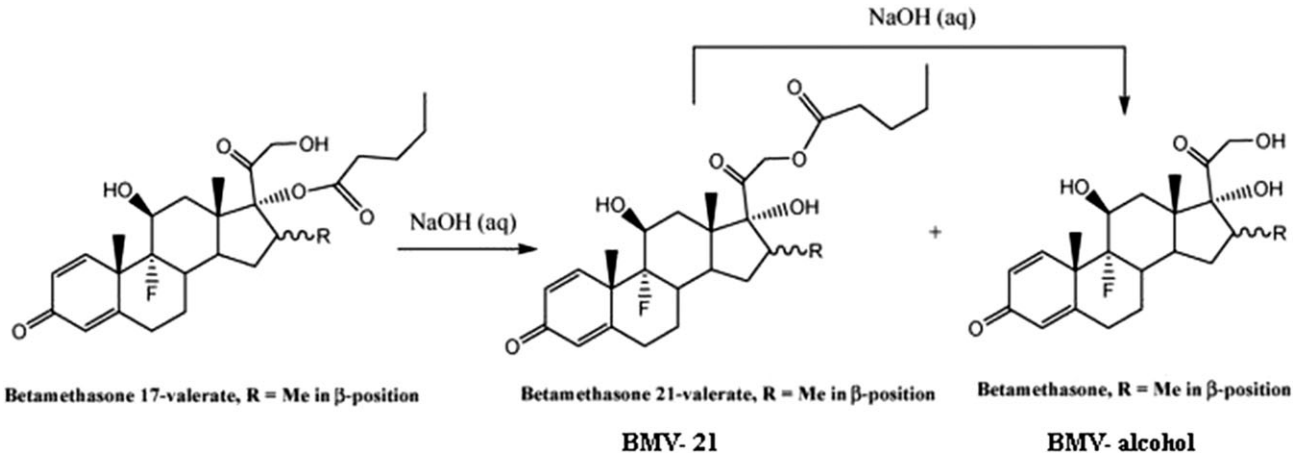


**Figure 4.** The suggested pathway for betamethasone valerate alkali induced degradation.

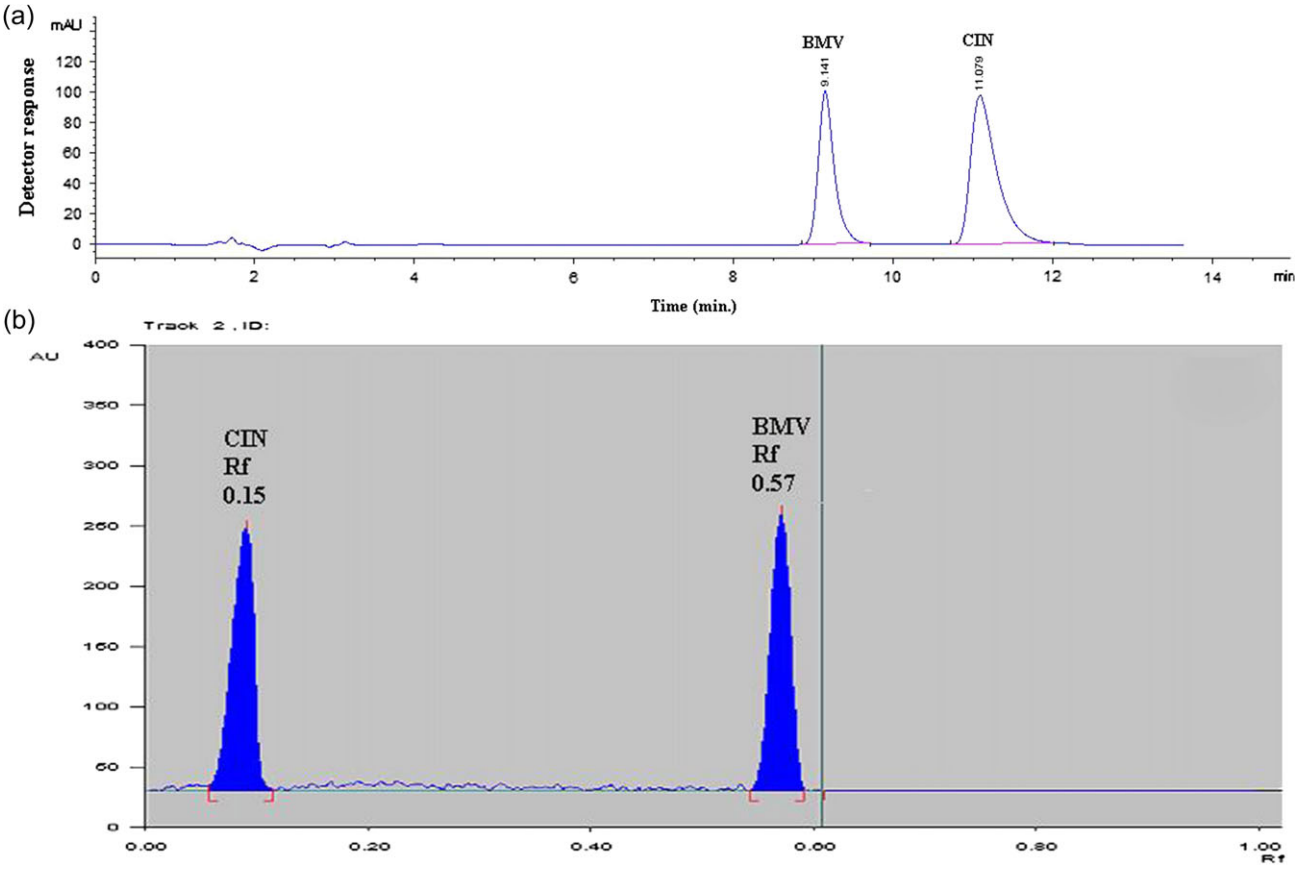


**Figure 5.** (a) High performance liquid chromatogram of the laboratory prepared mixture of CIN and betamethasone valerate (each 50.0 μg/mL). (b) Densitogram of the laboratory prepared mixture of cinchocaine hydrochloride and betamethasone valerate (each 1.0 μg/band).

## Development of the proposed HPLC and HPTLC Methods

Several trials were carried out for simultaneous separation of CIN and BMV in their binary mixture. Best results were achieved by applying the chromatographic conditions given in the experimental part where good baseline separation of the two drugs was obtained. Figure 5a shows high performance liquid chromatogram of CIN and BMV binary mixture. The obtained retention times were 11.1 min and 9.2 min, respectively. Peaks purity was assessed by DAD detector and it was found to be 999.99 and 999.98 for CIN and BMV, respectively. As for HPTLC method the two components were completely separated giving $R_f$ values of 0.15 and 0.57 for CIN and BMV, in order as shown in Figure 5b.

## Optimization of the chromatographic conditions

### For HPLC method

This study described an HPLC method for the simultaneous determination of CIN and BMV in the presence of their hydrolytic degradation products. Different mobile phase compositions with buffers of different pH values were tried to obtain good resolution between the studied drugs and their degradation products. The mobile phase of choice was found to be acetonitrile:acetate buffer (pH 6.5 ± 0.1) in a ratio of (55:45, v/v) with an isocratic elution mode and a flow rate of 1.2 mL/min. Decreasing the ratio of acetonitrile in mobile phase leads to delay and broadening of peaks, while its increase although it shortened the analysis time, yet it caused poor separation between the peaks. The pH of the mobile phase critically affected the retention time and separation of the compounds. The optimum pH was found to be 6.5 ± 0.1. By increasing the pH, CIN and BMV-21 were poorly separated.

The wavelength selected for detection was chosen to obtain the maximum sensitivity of the separated compounds. Based on the UV-absorption spectra of CIN and BMV; choosing 320 nm for CIN and its acid degradation products gave good results; but it could not detect BMV and its alkali induced degradation products. A wavelength 240 nm was selected to determine both drugs in addition to their corresponding degradates, also an advantage at this wavelength, BMV could be determined in small amounts as it represents its maximum wavelength.

Two peaks appeared upon applying the proposed HPLC method on separation of CIN acid degradation products solution, at retention times 1.6 and 2.2 min (Figure 6a).

The BMV alkali degradation solution showed two peaks by the proposed HPLC method at retention times 3.2 and 12.8 min, corresponding to BMV-alcohol and BMV-21, in order (Figure 6a).

### For HPTLC-densitometric method

Scan mode and wavelength of the detection were optimized to provide accurate and precise results for determination of the studied drugs. In trials to have the optimum separation, different developing systems of variable compositions and ratios were tried. The systems tried included a three-component system, ethyl acetate–methanol–ammonia in different ratios, but no good separation was achieved. Other four-component systems were tried; toluene–isopropanol–acetone–ammonia and benzene–acetone–methanol–ammonia in different ratios; where incomplete resolution of the drugs and their hydrolytic degradation products was obtained with highly tailed peaks. Finally, Chloroform–toluene–ethanol–acetic acid system was tried in different ratios to obtain optimum separation of the six components, namely; the two drugs and their degradation products. The obtained HPTLC chromatograms showed relatively good separation, and by slight adjustment of the

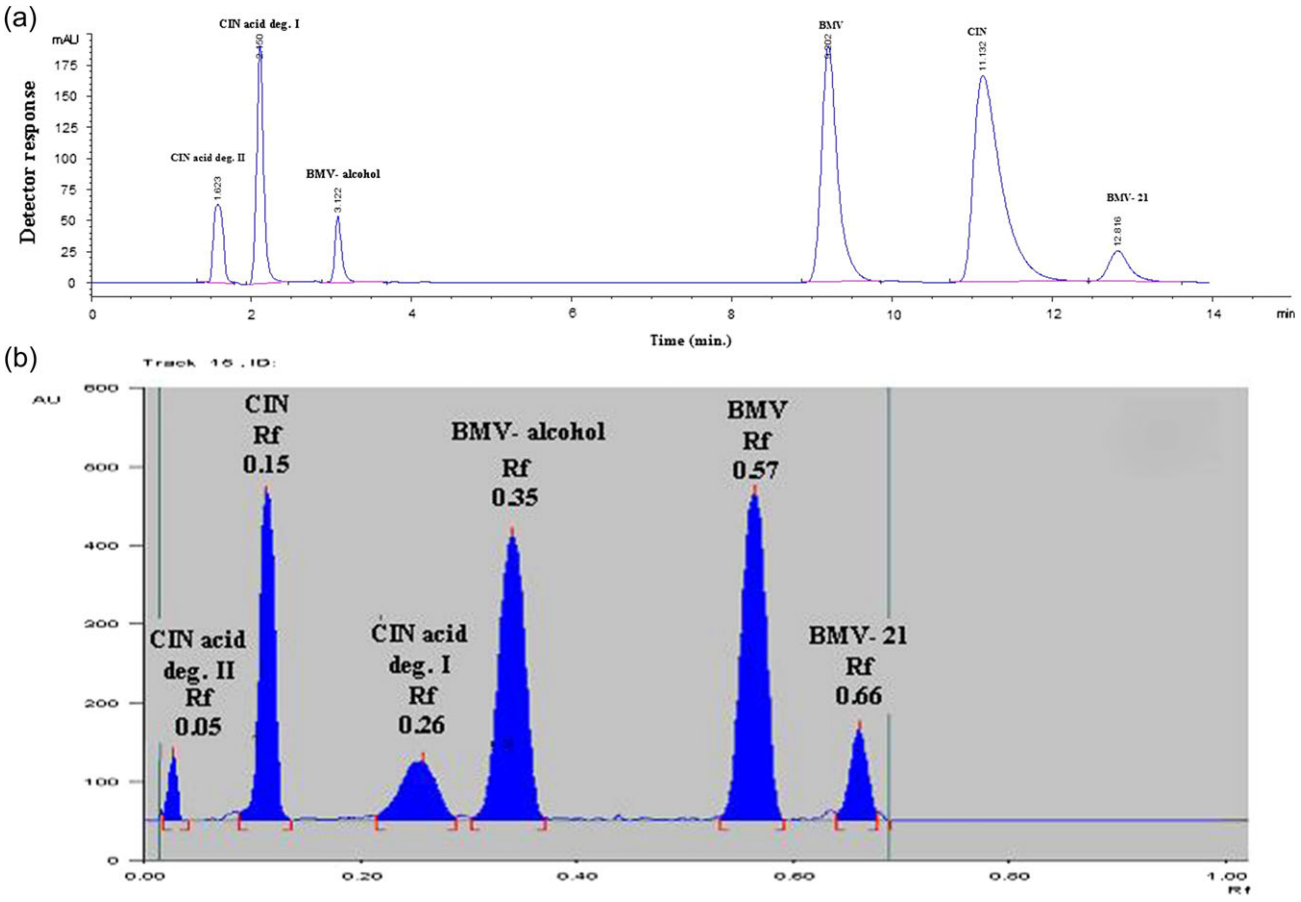


**Figure 6.** Selective separation of CIN and BMV in presence of their pooled degradation products showing: (a) high performance liquid chromatogram of laboratory prepared mixture of cinchocaine hydrochloride acid degradation products, betamethasone valerate alkali degradation products, betamethasone valerate and cinchocaine hydrochloride 70.0, 35.0, 100.0 and 100.0 μg/mL, respectively. (b) densitogram of laboratory prepared mixture of cinchocaine hydrochloride acid degradation products, betamethasone valerate alkali degradation products, betamethasone valerate and cinchocaine hydrochloride (each 2.50 μg/band).

ratios, a solvent of chloroform–toluene–ethanol–glacial acetic acid (4.5:4.5:1:1, by volume) could give the best results with minimum tailing and maximum separation as indicated by comparatively wide differences between $R_f$ values of the studied drugs and their degradation products as shown in Figure 6b. The wavelength of the scanning 240 nm could determine all the cited components with maximum sensitivity.

The proposed HPTLC method showed two bands at $R_f$ 0.05 and 0.26 for the two CIN acid degradation products; while for BMV the obtained two degradation products were as two bands at $R_f$ 0.35 and 0.66 (Figure 6b).

The two chromatographic methods were used to check complete acid degradation of CIN by disappearance of HPLC peak at 11.1 min and TLC band with $R_f$ at 0.15, corresponding to intact CIN. As for BMV complete degradation was confirmed by disappearance of HPLC peak at 9.2 min and TLC band with $R_f$ at 0.57, corresponding to intact BMV (Figure 5).

BMV was also tested for thermal stress conditions. The methanolic solution was subjected to heat at 80°C in a water bath and samples were taken every 30 min. It was found that BMV is liable to thermal degradation and yields BMV-21 after 2 h. The results were confirmed by comparing retention time and $R_f$ of the resulted degradation peaks with that of reference standards.

Successful separation of all components solution mixture of the two drugs and their degradation products was achieved by applying the optimum chromatographic conditions. For HPLC method, baseline separation and sharp peaks without tailing were obtained in the sequence of CIN acid degradate II, CIN acid degradate I, BMV-alcohol, BMV, CIN and BMV-21 were obtained at ≈ 1.6, 2.2, 3.1, 9.2, 11.1 and 12.8 min. However, very little practical deviations from the mean retention time values of the resolved drugs were observed at different days. For HPLC method all the six substances were separated within ≈ 15 min (Figure 6a). For HPTLC, the obtained $R_f$ values were in the sequence of CIN acid degradate II, CIN, CIN acid degradate I, BMV-alcohol, BMV and BMV-21 at 0.05, 0.15, 0.26, 0.35, 0.57 and 0.66 (Figure 6b).

In accordance to the USP (10); the proposed HPLC method system suitability parameters were calculated. The obtained values showed complying of the methods as for resolution, selectivity and peaks symmetry (Table I). Robustness of the method was assessed by deliberate variation of the wavelength ± 1 nm, buffer pH ± 0.1 and flow rate ± 0.1 mL/min and was expressed by % RSD.

System suitability parameters of the proposed HPTLC-densitometric method were calculated showing good resolution, selectivity and symmetrical peaks (11). Capacity factor ($k'$), tailing factor ($T$), selectivity factor ($\alpha$) and resolution ($R_s$), were calculated and are shown in Table II. Robustness of the method was tested by changing the detecting wavelength by ±1 nm, developing system volume, saturation time and time taken from chromatographic separation to scanning. The system suitability parameters did not change which assured the method robustness.

**Table I.** System Suitability Parameters of the Proposed HPLC Method

| Parameter | Obtained value | | | | | | |
|---|---|---|---|---|---|---|---|
| | CIN Acid Deg. II | CIN Acid Deg. I | BMV-alcohol | BMV | CIN | BMV-21 | Reference value |
| Capacity factor ($k'$)[a] | 0.25 | 0.65 | 1.40 | 6.08 | 7.56 | 8.86 | |
| Selectivity ($\alpha$)[b] | 2.60 | 2.15 | 4.34 | 1.24 | 1.17 | | >1 |
| Resolution ($R_s$)[b] | 2.67 | 4.44 | 15.75 | 3.57 | 1.85 | | $R_s$ >1.5 |
| Tailing factor ($T$)[a] | 1.12 | 0.95 | 1.09 | 1.10 | 1.17 | 1.12 | ≈1 |
| Number of theoretical plates ($N$) | 1024 | 1239 | 4096 | 4011 | 3600 | 11151 | Increase with efficiency of separation |
| Height equivalent to theoretical plates (HETP) | 0.024 | 0.020 | $6.10 \times 10^{-3}$ | $6.23 \times 10^{-3}$ | $6.90 \times 10^{-3}$ | $2.24 \times 10^{-3}$ | The smaller the value, the higher the column efficiency |
| Retention time (min ± 0.2) | 1.6 | 2.2 | 3.1 | 9.2 | 11.1 | 12.8 | |

[a]Calculated for each individual peak.
[b]Calculated for each of two successive peaks.

**Table II.** System Suitability Parameters of the Proposed HPTLC-densitometric Method

| Parameter | Obtained value | | | | | |
|---|---|---|---|---|---|---|
| | CIN Acid Deg. II | CIN | CIN Acid Deg. I | BMV-alcohol | BMV | BMV-21 |
| Retardation factor ($R_f$) | 0.05 | 0.15 | 0.26 | 0.35 | 0.57 | 0.66 |
| Capacity factor ($K'$) | 19.00 | 5.67 | 2.85 | 1.86 | 0.75 | 0.52 |
| Selectivity ($\alpha$)* | 3.35 | 1.99 | 1.53 | 2.48 | 1.44 | |
| Resolution ($R_s$)* | 3.71 | 3.38 | 1.50 | 5.23 | 2.73 | |
| Tailing factor ($T$) | 1.16 | 0.92 | 0.91 | 1.08 | 1.00 | 1.00 |

*Calculated for each of two successive peaks.

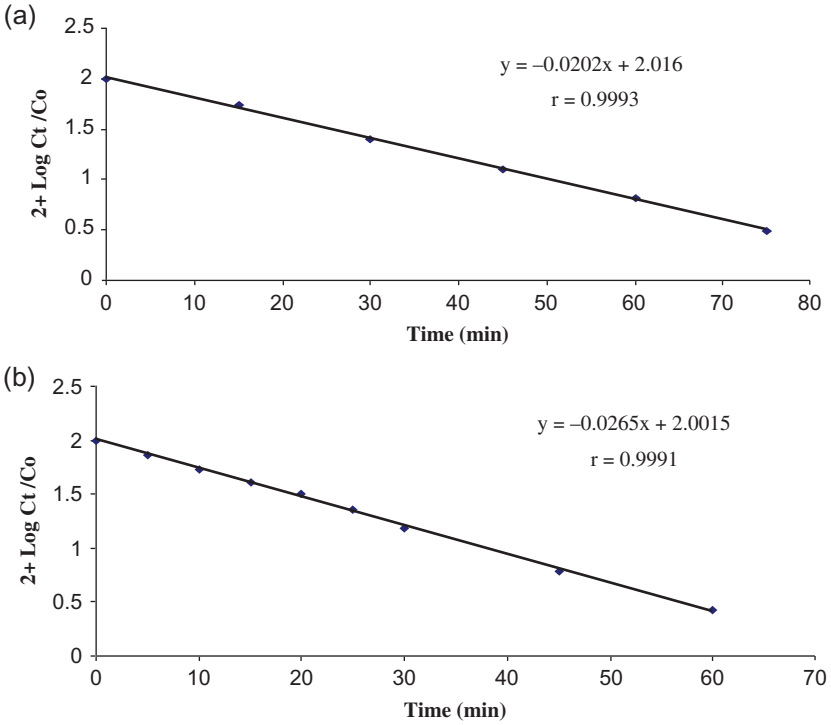


**Figure 7.** Pseudo first-order plot for: (a) acid degradation of CIN using 2M HCl at 100°C. (b) alkali degradation of BMV using 0.08M NaOH at ambient temperature.

**Table III.** Validation of the Proposed HPLC and HPTLC-densitometric Methods for the Determination of Cinchocaine Hydrochloride and Betamethasone Valerate

| Method Parameter | HPLC method | | HPTLC-Densitometric method | |
|---|---|---|---|---|
| | CIN | BMV | CIN | BMV |
| Range | 4–300 (μg/mL) | 4–350 (μg/mL) | 0.5–12 (μg/band) | 0.5–10 (μg/band) |
| Regression equations parameters | | | | |
| Slope (b)[a] | 44.649 | 29.187 | | |
| Coefficient 1 (b1)[b] | | | −85.822 | −150.07 |
| Coefficient 2 (b2)[b] | | | 2809.3 | 3037.6 |
| Intercept (a)[a,b] | 26.94 | 19.581 | 1756.3 | 1474.8 |
| Correlation coefficient (*r*) | 0.9999 | 0.9999 | 0.9999 | 0.9999 |
| Accuracy (Mean ± SD) | 100.09 ± 1.25 | 98.98 ± 0.30 | 99.99 ± 0.61 | 99.85 ± 0.95 |
| Specificity[c] | 100.25 ± 1.46 | 100.04 ± 0.98 | 100.24 ± 0.90 | 100.38 ± 1.43 |
| Precision | | | | |
| (% RSD)[d] | 0.82 | 0.76 | 0.40 | 0.25 |
| (% RSD)[e] | 1.37 | 1.45 | 1.63 | 1.55 |
| Robustness[f] | 1.56 | 1.26 | 1.12 | 1.73 |

[a]Regression equation for HPLC: $A = a + bc$, where '*A*' is the area and '*c*' is the concentration.

[b]Coefficients 1 and 2 are the coefficients of *X* and $X^2$, respectively. Following a polynomial regression: $A = b1c^2 + b2c + a$, where '*A*' is the peak area, '*c*' is the concentration of CIN and BMV (μg/band), 'b1' and '*b2*' are coefficients 1 and 2, respectively and '*a*' is the intercept.

[c]Recovery of CIN and BMV in laboratory prepared mixtures containing degradation products.

[d]Intraday precision [average of three different concentrations of three replicate each ($n = 9$) within the same day].

[e]Interday precision [average of three different concentrations of three replicate each ($n = 9$) repeated on three successive days].

[f]Robustness; % RSD (average of three different concentrations of three replicate each ($n = 9$) analyzed in different conditions mentioned before).

### Kinetic studies

The developed HPLC method was used to study the kinetics of the acid induced degradation of CIN and alkali degradation of BMV by estimating the concentration of the remaining drug at different time intervals. Figure 7 shows a plot of the log percentage of remaining drug against time. The linear relationship obtained indicates pseudo-first order reaction kinetics for both acid and alkali degradation. For CIN acid degradation, the observed rate constant was 0.0465/min while its $t½$ value was 14.9 min. For alkali degradation of BMV, the rate constant was 0.0610/min and its $t½$ value was 11.4 min. However, kinetic acid degradation study of CIN was previously mentioned but using first derivative spectrophotometic

**Table IV.** Determination of Cinchocaine Hydrochloride and Betamethasone Valerate in Laboratory Prepared Mixtures by the Proposed HPLC and HPTLC-Densitometric Methods

| % Degradation | HPLC | | | | | | HPTLC-densitometric | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration (μg/mL) | | | | Found % Recovery[a] | | Concentration (μg/band) | | | | Found % Recovery[a] | |
| | CIN | BMV | CIN acid degradates | BMV alkaline degradates | CIN | BMV | CIN | BMV | CIN acid degradates | BMV alkaline degradates | CIN | BMV |
| 0[b] | 50 | 10 | | | 99.34 | 100.70 | 5 | 1 | – | – | 99.80 | 101.00 |
| 10 | 90 | 45 | 10 | 5 | 99.36 | 99.96 | 4.50 | 4.50 | 0.50 | 0.50 | 101.56 | 99.56 |
| 30 | 70 | 35 | 30 | 15 | 101.00 | 100.20 | 3.50 | 3.50 | 1.50 | 1.50 | 101.14 | 102.00 |
| 50 | 50 | 25 | 50 | 25 | 98.28 | 98.60 | 2.50 | 2.50 | 2.50 | 2.50 | 99.60 | 100.40 |
| 70 | 30 | 15 | 70 | 35 | 101.73 | 99.40 | 1.50 | 1.50 | 3.50 | 3.50 | 99.33 | 101.33 |
| 90 | 10 | 5 | 90 | 45 | 101.80 | 101.40 | 0.50 | 0.50 | 4.50 | 4.50 | 100.00 | 98.00 |
| Mean | | | | | 100.25 | 100.04 | | | | | 100.24 | 100.38 |
| ± SD | | | | | 1.46 | 0.98 | | | | | 0.90 | 1.43 |

[a]Average of three determinations.
[b]Ratio of CIN and BMV in pharmaceutical formulation.

**Table V.** Determination of Cinchocaine Hydrochloride and Betamethasone Valerate in Pharmaceutical Dosage Form by the Proposed Methods and Application of the Standard Addition Technique

| Product | Drug | Standard addition | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HPLC | | | | HPTLC-densitometry | | | |
| | | Recovery% ± SD of the claimed amount[a] | Taken (μg/mL) | Added (μg/mL) | Recovery% from the added amount[a] | Recovery% ± SD of the claimed amount[a] | Taken (μg/band) | Added (μg/band) | Recovery% from the added amount[a] |
| Supraproct-S® Ointment. Each 1 g was labeled to contain 5 mg CIN and 1 mg BMV, batch no. 0014. | CIN | 99.83 ± 1.42 | 50.00 | 25.00 | 98.20 | 99.47 ± 0.65 | 2.00 | 1.00 | 99.00 |
| | | | | 50.00 | 100.80 | | | 2.00 | 100.00 |
| | | | | 100.00 | 99.06 | | | 4.00 | 100.25 |
| | | | Mean ± SD | | 99.35 ± 1.32 | | Mean ± SD | | 99.75 ± 0.66 |
| | BMV | 100.53 ± 1.06 | 10.00 | 5.00 | 98.60 | 101.14 ± 0.41 | 1.00 | 0.50 | 100.00 |
| | | | | 10.00 | 100.10 | | | 1.00 | 101.00 |
| | | | | 20.00 | 101.00 | | | 2.00 | 101.50 |
| | | | Mean ± SD | | 99.90 ± 1.21 | | Mean ± SD | | 100.83 ± 0.76 |

[a]Average of three determinations.

method and was also found to follow pseudo-first order reaction (4).

ICH guidelines (12) validation parameters were followed and performed for the two proposed methods. The obtained range for CIN and BMV was 4–300 and 4–350 μg/mL, respectively, for the HPLC method using linear regression equation; while for HPTLC-densitometric method, polynomial equation was applied; the obtained range was 0.5–12 and 0.5–10 μg/band for CIN and BMV, respectively. Accuracy and precision results of the two proposed methods and the assessment of their robustness with respect to the effect of small but deliberate variation in chromatographic conditions are shown in Table III. The characteristic parameters for the regression equations of the proposed methods were also given in Table III. The proposed HPLC and HPTLC-densitometric methods were selective and accurate for determination of the investigated drugs in laboratory prepared mixtures (Table IV).

CIN and BMV in pharmaceutical formulation, Supraproct-S® ointment, were analyzed by the suggested methods; the results are given in (Table V). Due to the great difference in polarity between CIN and BMV, a solvent mixture of acetonitrile: water (50:50, v/v) was found to be optimum for extracting both components from their dosage form. This approach gave the better percentage extracting recoveries without interference from other excipients than upon using a single extracting solvent.

Standard addition technique was used to assess the validity of the proposed methods. The accurate results obtained revealed that there was no interference from excipients as shown in Table V.

Table VI presents the statistical comparison of the results of the pure compounds analysis by the proposed methods and that of the official ones, with 95% confidence level there is no significant difference between the proposed and the official methods with regard to accuracy and precision by calculating Student's *t* test and the *F*-ratio.

## Discussion

Pharmaceutical impurities are components found in a drug substance or drug product that are neither the drug substance nor excipients according to International Conference on Harmonization (ICH) guidelines (9). These could be related compounds or degradation products of the intact drugs. Development of stability indicating, accurate and precise assay methods (SIAMs) is one of the important requirements to be fulfilled for method development

**Table VI.** Statistical Comparison of the Results Obtained by the Proposed Methods and Those Obtained by the Official Ones for the Analysis of Cinchocaine Hydrochloride and Betamethasone Valerate in their Pure Forms

| Value | HPLC | | HPTLC-densitometry | | Official method (1) | |
|---|---|---|---|---|---|---|
| | CIN | BMV | CIN | BMV | CIN[a] | BMV[b] |
| Mean | 100.16 | 99.41 | 99.98 | 100.02 | 100.05 | 100.03 |
| SD | 1.43 | 0.78 | 0.64 | 0.95 | 1.32 | 1.17 |
| % RSD | 1.43 | 0.78 | 0.64 | 0.95 | 1.32 | 1.17 |
| n | 6 | 6 | 7 | 6 | 5 | 5 |
| Variance | 2.04 | 0.61 | 0.41 | 0.90 | 1.74 | 1.37 |
| Student's *t* test | 0.132 (2.262)[c] | 1.012 (2.262)[c] | 0.110 (2.228)[c] | 0.015 (2.262)[c] | | |
| *F* value | 1.17 (6.26)[c] | 2.25 (5.19)[c] | 4.24 (4.53)[c] | 1.52 (5.19)[c] | | |

[a]For cinchocaine hydrochloride: titrimetric method against 0.1M NaOH using a mixture of 0.01M HCl and alcohol as a solvent with potentiometric detection of end point.

[b]For betamethasone valerate: spectrophotometric determination at 240 nm against 96% ethanol as blank, taking the specific absorbance to be 325 nm.

[c]The values in the parenthesis are the corresponding theoretical values of *t* and *F* at $P = 0.05$.

according to ICH. It was worthwhile to investigate the degradation of the studied drugs. Both drug substances are susceptible to hydrolytic degradation. CIN acid degradation products were separated and identified by Mass Spectra. While, BMV alkali degradation products were confirmed by comparing retention time and $R_f$ of the resulted degradation peaks with that of reference standards.

In this work, stability-indicating HPLC and HPTLC-densitometric methods were developed for the determination of CIN and BMV in presence of their degradation products (i.e., impurities). Both methods could, simultaneously, determine the two drugs in the presence of their hydrolytic degradation products without any interference. Critical conditions affecting the separation, such as solvent type, type and pH of the used buffer, organic solvent ratio in mobile phase and flow rate, have been carefully studied. Satisfactory system suitability parameters of resolution efficiency were obtained for the proposed HPLC-DAD and HPTLC methods (Tables I and II). The developed methods were validated in accordance to the ICH guidelines to evaluate adequate validation characteristics (Table III). The results for determination of CIN and BMV as prepared synthetically in laboratory mixtures and in the presence of their hydrolytic degradation products ascertained the specificity of the proposed chromatographic methods (Table IV). The proposed methods were successfully applied for determination of CIN and BMV in Supraproct-S® ointment after the proper selection of the optimum extracting solvent. The standard addition technique ascertained the validity of the proposed methods (Table V). The results obtained from the proposed chromatographic methods were statistically analyzed and compared with those obtained by applying the official methods for both drugs, showing no significant difference regarding both accuracy and precision, as the *t*-value and *F*-value were less than the theoretical ones (Table VI). Meanwhile, HPLC-DAD method was used to study the kinetics of acid and alkali induced degradation of CIN and BMV.

## Conclusion

The proposed methods were used for simultaneous determination of CIN and BMV in pure forms and pharmaceutical formulation; meanwhile they are stability-indicating ones. Both CIN and BMV could be determined without any interference from the excipients and in the presence of their hydrolytic degradation products, which are also considered as potential impurities. The developed methods were validated and could be used for routine analysis in quality control laboratories and for further kinetic studies where economy and time are essential.

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