

LC-MS as a Stability-Indicating Method for Analysis of Hyoscine N-Butyl Bromide under Stress Degradation Conditions with Identification of Degradation Products

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Abstract

Hyoscine N-Butyl Bromide (HBB) was subjected to different ICH prescribed stress conditions. It showed extensive decomposition under base hydrolytic conditions, while it was less liable to stress acid hydrolytic conditions. It showed also moderate degradation in response to oxidation stress of hydrogen peroxide. The drug showed no changes under photolysis conditions.

In total, a number of major degradation products were detected by HPLC and identified by LC-MS. For establishment of stability-indicating assay, the reaction solutions in which different degradation products were formed were prepared, and the separation was optimized by varying the HPLC conditions. An acceptable chromatograms was achieved using a C18 column using (water: methanol 50: 50 v/v, pH adjusted to 3.9 with trifluoroacetic acid) as a mobile phase with flow rate of 1.0 ml min⁻¹ and UV detection wavelength at 210 nm. The percent of degradation was calculated in each run by measuring the intensity of the peak area of the intact drug at 6.2 min. Complete degradation only occur in case of 5 N NaOH indicates that the drug is very sensitive to alkaline hydrolysis.

The LC-MS study was carried out to identify the major degradation products using a sunfire (waters) C-18 column and a mobile phase comprising of acetonitrile: 0.1M ammonium acetate (80:20, v/v) with flow rate of 1.0 ml min⁻¹. MS measurements were acquired in positive ion full scan modes from 50 to 400 amu. The *m/z* values of the main peaks were investigated with the expected chemical structure of degradates.

Keywords: Hyoscine N-Butyl Bromide; Stress degradation; Stability-indicating method; LC-MS; Degradation products

Introduction

Hyoscine N-Butyl Bromide is a quaternary ammonium anticholinergic agent. It has antispasmodic action on the smooth muscles of the gastrointestinal, biliary, and urinary tracts [1]. Its chemical name is (-)-(1S,3s,5R,6R,7S,8r)-6,7-Epoxy-8-butyl-3-[(S)-tropoyloxy]tropanium bromide [1] (Figure 1). HBB is a white, crystalline powder or colorless crystals, efflorescent, freely soluble in water, soluble in alcohol. It melts at about 139°C-141°C [2]. The empirical formula of HBB is C₂₁H₃₀BrNO₄ and its molecular weight is 440.4 and 360.1 without Bromide atom.

Many analytical techniques have been reported in the literature for determination of HBB in pharmaceutical preparations using spectrophotometric methods [3-5], chromatographic methods [6-8], electrochemical methods [8-11], Capillary electrophoresis methods [12,13] and titrimetric method [14].

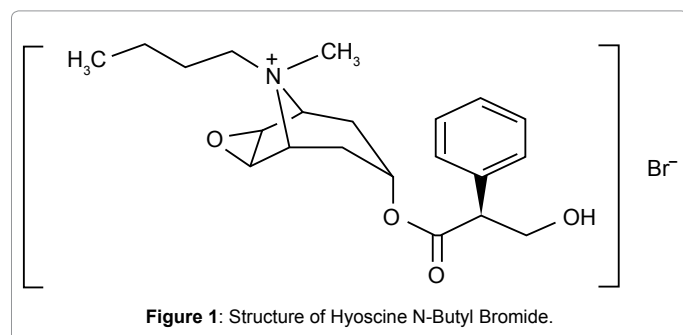
LC is a common technique. LC may be coupled with UV detection or mass spectrophotometry. Mass spectrophotometer is the most powerful analytical technique. LC-MS is a hyphenated technique; combing separation power of HPLC, with the great detection power of mass spectrometry. LC-MS provides online molecular masses identification without need of prior isolation.

LC-MS has many applications. It can be used for detection of low concentration of drug or poison, impurities profile, metabolite studies, bioequivalent and bioavailability studies.

Recently, LC-MS has been used for identification of degradation products for many drugs in stress conditions as Prulifloxacin [15], lornoxicam [16], Isoniazid [17] and Eletriptan hydro bromide [18].

Pharmaceutical drugs must be tested with a stability-indicating method before release to market according to good manufacturing practices.

So far to our present knowledge neither LC-MS nor HPLC



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stability-indicating analytical method for HBB has been published in the literature, although a UPLC/MS-MS method for determination of the drug in plasma has been published [8]. However this method does not discuss the identity of the degradation products and does not offer a stability study. The present work is dealing with HPLC as stability indicating method, developing forced degradation studies according to ICH guidelines and discussing probability of formed degradates with LC-MS (Table 1).

Experimental

Samples

Hyoscine N-Butyl Bromide (HBB) was kindly supplied by CID Co. Chemical Industries Development, Giza, Egypt. Its purity was found to be 99.21 ± 0.602 according to the company analysis certificate (HPLC).

Chemical and reagents

All chemicals and solvents were of analytical grade and were used without further purification.

- Hydrochloric acid, Orthophosphoric acid, glacial acetic acid,

Sodium hydroxide, hydrogen peroxide and ammonium acetate all are from (El - Nasr Pharmaceutical Chemicals Co, Abu-Zabaal, Cairo, Egypt).

- Acetonitrile (E.Merck, Germany).Methanol HPLC grade (Sigma Aldrich, Germany).
- Deionised water (SEDICO pharmaceutical Co., 6th October City, Egypt).

Trifluoroacetic acid from Spectrochem, India.

Instrumentations

- Shimadzu Class - LC 10 AD Liquid Chromatography supplied with Shimadzu SPD - 10 A UV - VIS Detector (Shimadzu Corporation, Japan). Phenomenex C18 (25 cm×4.6 mm i.d, 5 μm particle size) column was used as a stationary phase for HPLC determinations (USA).
- The LC system is integrated system shimadzu controller (CBM20Alite). The system consist of pump (Shimadzu LC20AD), auto injector (Shimadzu SIL20A), the device is

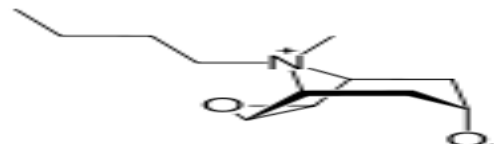

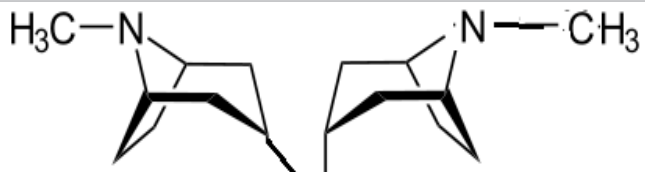
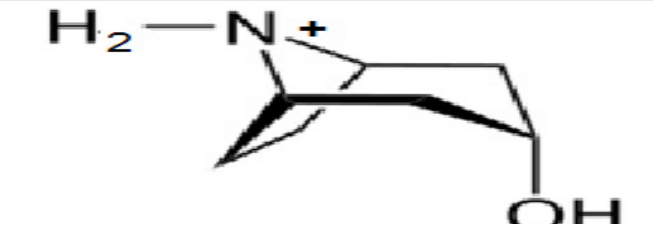
Name	Molecular formula	Structural form and chemical name	Molecular weight
Degradate I	$C_{12}H_{21}O_2N$	 <p>9-Butyl-7-hydroxy-9-methyl-3-oxa-9-azonia-tricyclo[3.3.1.0^{2,4}]nonane</p>	211.3
Degradate II	$C_8H_{15}ON$	 <p>8-Methyl-8-aza-bicyclo[3.2.1]octan-3-ol</p>	141.2
Degradate III	$C_{16}H_{28}ON_2$	 <p>3-(8-methyl-8-aza-bicyclo[3.2.1]octan-2-yl)oxy-8-methyl-8-aza-bicyclo[3.2.1]octane</p>	264.4
Degradate IV	$C_7H_{14}ON$	 <p>3-Hydroxy-8-azonia-bicyclo[3.2.1]octane</p>	128.2

Table 1: Expected Chemical structures of the scanned compounds and their masses values.

Triple Quadrupole LC/MS/MS Mass Spectrometer (API 3200). The chromatographic separations were carried out on a sunfire[®] from waters C-18 (50 mm×4.6 mm i.d., particle size 5 μm) column.

- Sonix TV ss-series ultrasonicator (USA).

Preparation of samples for HPLC and LC-MS analyses

A sample concentration of 1000 μg ml⁻¹ was used to conduct degradation studies. The degradation samples were injected directly without neutralization (in case of acid and base hydrolysis) and diluted suitably to a concentration of 2.5 μg ml⁻¹ for analysis by LC-MS while degradation samples for HPLC were injected directly without any dilution. All the solutions were filtered using 0.22 micron membrane filter before HPLC and LC-MS injections.

Forced degradation studies

Stress studies were carried out under ICH prescribed stress conditions [19].

Forced degradation was carried out under stress conditions as follow

- ✓ For photolysis conditions (direct sunlight for 12 hours).
- ✓ For acidic conditions (0.1 N hydrochloric acid with reflux for 12 hours) and (5 N hydrochloric acid with reflux for 3 hours).
- ✓ For basic conditions (0.1N sodium hydroxide with reflux for 12 hours) and (5N sodium hydroxide with reflux for 3 hours).
- ✓ For oxidative conditions (3.0 % H₂O₂ for 12 hours) and (30.0 % H₂O₂ for 12 hours).

Degradation studies in acidic and basic media were carried out at 80°C. After the different treatments were completed, all the solutions and blanks (solutions without any treatment) were filtered with a 0.45 μm syringe filtration disk to the vials for injection in HPLC and LC-MS systems. The purpose of the LC-MS method is to scan masses of HBB and its degradation products (Table 2).

Linearity and construction of calibration curve for HBB by HPLC method

Accurate aliquots equivalent to (20-500) μg of HBB were transferred from its corresponding working solutions (100 μg.mL⁻¹) into a set of a series of 10-mL volumetric flasks. The volume was completed with methanol. Triplicate 20 μL injections were made for each concentration. The separation was done on a C₁₈ column using (water: methanol 50: 50 v/v, pH adjusted to 3.9 with trifluoroacetic acid) as a mobile phase. All solvents were filtered through a 0.45 μm membrane filters before use and degassed in an ultrasonic bath for 20 min. Record the chromatograms at ambient temperature maintaining the flow rate at 1.0 mL.min⁻¹ and detect the effluent at 210 nm. Construct the calibration curves for HBB

by plotting the peak area/ 10⁴ versus the corresponding concentration and then compute the regression equation.

LC-MS conditions and Characterization of degradation product(s)

First, the reaction solutions were individually subjected to LC-MS. The studies were conducted using a mobile phase composed of (acetonitrile: 0.1 M ammonium acetate 80:20, V/V) with a flow rate of 1 mL.min⁻¹ with isocratic flow. LC-MS studies were carried out in Turbo spray ionization (TSI), scan type is Q1 Multiple Ions (Q1 MI). MS measurements were acquired in positive ion full scan modes from 50 to 400 amu. The LC-MS method can be used for characterization of both the degradation products and the intact drug. LC-MS studies were performed to determine *m/z* values of the major degradation products formed under various stress test conditions. The obtained values were compared with the molecular weights of expected degradation products of HBB.

Results and Discussion

Simple, selective, sensitive and accurate isocratic HPLC method was adopted for the determination of HBB in presence of degradation products without prior separation. Mass spectrum scanning by LC-MS was developed for determination of degradation behavior of HBB and Characterization of degradation product(s).

A satisfactory separation in HPLC method was obtained by using C18 (25 cm×4.6 mm i.d. 5 μm particle size) column as a stationary phase and using (water: methanol 50:50, v/v pH adjusted to 3.9 with CF₃COOH acid) as a mobile phase, maintaining the flow rate at 1.0 mL min⁻¹ with UV detection at 210 nm. The retention time for HBB was 6.2 min, Figure 2 (Data included as supplementary).

HPLC method validation

Method validation was performed according to ICH guidelines [19].

Linearity of the proposed method was evaluated and it was evident in the concentration range of 2-50 μg.mL⁻¹ for HBB. Good linearity was evident by the high value of the correlation coefficient and the low intercept value, (Figure 3) and (Table 3). The regression equations were calculated and found to be:

$$Y = 2.507 C - 0.409 \quad r = 0.9997$$

Where Y is the peak area /10⁴, C is HBB concentration in μg. mL⁻¹ and r is the correlation coefficient.

Precision of the proposed HPLC method was evident as shown in Table 3.

Degradation Studies	Time	Assay of HBB % of peak area	Remarks
1-Blank		99.00	No significant
2- Photolytic Condition	12 h	99.02	degradation observed
3-Base hydrolysis (0.1 N NaOH at 80°C)	12 h	25.00	Degradation products I and II observed
4- Base hydrolysis (5 N NaOH at 80°C)	3 h	0.00	Degradation products I , II and III observed.
5-Acid hydrolysis (0.1 N HCl at 80°C)	12 h	99.00	No significant
6- Acid hydrolysis (5 N HCl at 80°C)	3 h	65.00	degradation observed
7-Oxidation by 3 %H ₂ O ₂ at 25 °C	12 h	90.00	Degradation product II
8- Oxidation by 30 %H ₂ O ₂ at 25 °C	3 h	72.00	observed unidentified Degradation products observed Degradation product IV Observed

Table 2: Mass balance for HBB drug substance in presence of degradation products formed during forced degradation studies.

Accuracy of the proposed method was checked by applying the proposed method for determination of different samples of standard HBB. The concentrations were calculated from the corresponding regression equation. The results obtained as shown in Table 4.

Specificity of the proposed method is evident HPLC chromatograms in Figure 2.

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as whole. System suitability is used to ensure system performance before or during the analysis of the drugs. System suitability was checked by calculating the capacity factor (K'), tailing factor(T), column efficiency (N) and resolution(Rs), where the system was found to be suitable as shown in Table 5.

Degradation behavior and Characterization of degradation product(s) according to HPLC chromatograms

HPLC chromatograms at 210 nm for all samples of standard drug, acid degradation, base degradation, oxidative degradation and photolysis are shown in Figure 3. The percent of intact HBB was calculated according to peak area of HPLC chromatograms as shown in Table 2.

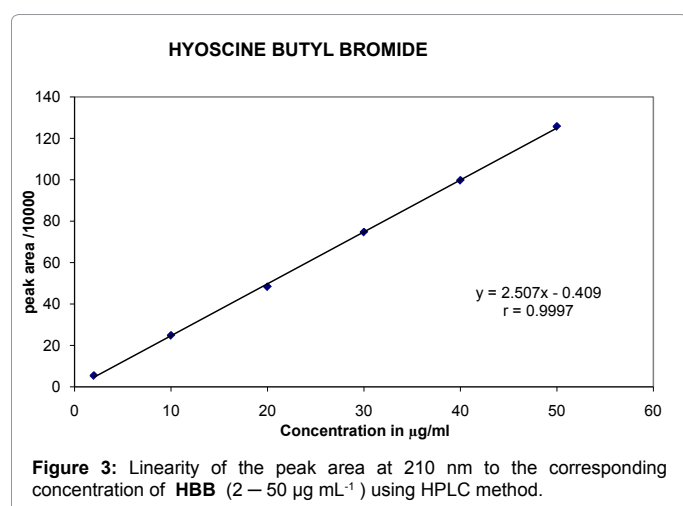
Base Hydrolysis: Appearance of new peaks in base hydrolysis with 0.1N NaOH at $R_t=2.9, 5.4$ min and reduction of peak area of HBB ($R_t=6.2$ min) indicate significant degradation of the drug in slightly basic medium (about 75% degradation).

Similarly, Appearance of new broad peak in base hydrolysis with 5 N NaOH at $R_t=3.4$ min and complete disappearance of HBB peak ($R_t=6.2$ min) indicate complete degradation of the drug in highly basic medium.

The retention times of degradates in 0.1 N NaOH differ from those of 5 N NaOH, this indicates that degradation products differ according to strength of the used base. This result matches with different mass spectra obtained for degradation in 0.1 N NaOH and 5 N NaOH.

Low concentration of the base (0.1N NaOH) is not enough for complete degradation of the drug even in longer time of reflux (about 12 hours).

Acid hydrolysis: No HPLC chromatogram changes appear in 0.1 N HCl chromatogram; this indicates that no significant degradation occurs in slightly acidic medium.



Parameters	HBB
Range ($\mu\text{g}\cdot\text{mL}^{-1}$)	2-50 ($\mu\text{g}\cdot\text{mL}^{-1}$)
Slope	2.507
Intercept	-0.409
Correlation coefficient (r)	0.9997
Accuracy (mean \pm SD)	100.20 \pm 0.806
(RSD%) ^{a*}	0.693
(RSD%) ^{b*}	0.716

(RSD%)^{a*}, (RSD%)^{b*} the intra-day and inter-day relative standard deviations of the average of concentrations (20, 40 and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ for each)

Table 3: Results of assay validation parameters of HPLC for the determination of HBB.

Taken ($\mu\text{g mL}^{-1}$)	Found* ($\mu\text{g mL}^{-1}$)	Recovery %
2.00	2.03	101.50
10.00	10.09	100.90
20.00	19.95	99.75
30.00	29.92	99.73
40.00	39.80	99.50
50.00	49.90	99.80
Mean \pm SD		100.20 \pm 0.806

*Average of three determinations

Table 4: Results of accuracy for determination of pure authentic of HBB by the proposed HPLC method.

Parameters	Obtained value for HBB	Reference value
Resolution (R_s)	4.11	>1.5
Capacity factor(K')	1.48	1- 10 acceptable
Tailing factor (T)	1.20	< 1.5-2
Number of Theoretical plate(N)	5082975	Increases with increases efficiency
HETP ($\text{cm}\cdot\text{plate}^{-1}$) Height equivalent to theoretical plate	0.4918	The smaller the value, the higher the efficiency

Table 5: Statistical analysis of parameters required for system suitability testing of HPLC method.

Decrease of peak area of HBB at $R_t=6.2$ min in chromatogram of 5N HCl than that of chromatogram of 0.1 N HCl and appearance of new peaks in acid hydrolysis with 5N HCl at $R_t=3.3, 9.8, 10.3$ min indicate that percent of degradation in acidic medium is greatly increased by increasing the concentration of the acid (about 35% degradation).

Oxidation by H_2O_2 : Decrease of peak area of HBB in chromatogram of 30 % H_2O_2 than that of chromatogram of 3 % H_2O_2 indicates that percent of degradation is greatly increased by increasing the strength of used H_2O_2 .

About 10% degradation was observed in oxidation by 3% hydrogen peroxide with formation of new peak at 13.3 min while 28% decomposition occurred in oxidation by 30% hydrogen peroxide with formation of new peaks at 9.3 and 10.3 min.

Photolysis: Evidently, the complete similarity between chromatograms of standard and photolysis sample indicates that light has no effect on HBB stability.

Degradation behavior and characterization of degradation product(s) according to mass spectrum scanning

In total, four major degradation products were detected by MS measurements on decomposition of the drug under various stress conditions (Table 1). The degradation behavior of the drug in individual stress conditions is discussed below according to mass

spectrum scanning, (Figure 4 data included as supplementary). Types of formed degradates in each stress degradation condition discussed collectively in Table 2.

Base hydrolysis: The drug degraded to great extent within 12 hours on heating at 80°C in 0.1N NaOH, forming mainly degradate I (212 m/z) and to less extent degradate II (142 m/z). This indicates the cleavage of ester bond between tropic acid and N butyle oxy tropine base.

The drug degraded completely upon hydrolysis of HBB after reflux with 5 N NaOH for 3 hours at 80°C but forming different degradates mainly degradate I (212 m/z) and to moderate extent degradate III (268 m/z). This indicates the cleavage of ester bond between tropic acid and N butyle oxy tropine base and also formation of dimer of tropine base through formation of ether bond.

Acid Hydrolysis: The drug was stable with 12 hours on heating at 80°C in 0.1 N HCl. This indicates that HBB resist acid hydrolysis and remain stable in the acidic juice of stomach so the drug is valid for oral route of administration.

Different results obtained upon reflux with 5 N HCl for 3 hours at 80°C as the drug degraded by 35% to form mainly degradate II (142 m/z). This indicates the cleavage of ester bond and formation of tropine base.

Oxidation by H₂O₂: The drug was relatively stable to 3% hydrogen peroxide at room temperature for 12 hours and about 10% degradation was observed with formation of two unidentified new degradate of (206.2 and 153.3 m/z). However, 28% decomposition occurred in 30% hydrogen peroxide, resulting in formation of mainly degradate IV of (130.4 m/z).

Photolysis: The LC profiles of light exposed drug samples in methyl alcohol were similar to that in the dark, indicating that light had no particular influence on the drug in solution.

Conclusions

It was possible in this study to develop a stability-indicating LC-MS method for HBB with identification of degradation products of HBB by subjecting the drug to ICH recommended stress conditions. The drug got well separated from degradation products by HPLC and the degradates were identified from each other in mass spectra of LC-MS.

The method is proved to be simple, specific, stability indicating and valid for the routine analysis of HBB in bulk drug form or in pharmaceutical formulations.

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