RP-HPLC Stability-Indicating Assay Method for Talinolol and Characterization of its Degradation Products

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Abstract

A reversed-phase high-performance liquid chromatographic method is developed and validated for the quantitative determination of talinolol and to characterize its degradation products. A very good resolution between peaks is achieved using a C18 column at 40°C. The mobile phase comprises of a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer (pH 4.4) in the ratio of 27:73 (v/v). The method is validated with respect to linearity, accuracy, precision, robustness, and forced degradation studies, which further proved the stability indicating power. During the forced degradation studies, talinolol is observed to be labile to hydrolytic stress and thermal stress (in the solution form). However, it is stable to the oxidative, photolytic, and thermal stress (in the solid form). The degraded products formed are investigated by electrospray ionization (ESI), time-of-flight mass spectrometry, nuclear magnetic resonance, and infrared spectroscopy. A possible degradation pathway is outlined based on the results. The method is found to be sensitive with a detection limit of 0.125 µg/mL and a quantitation limit of 0.378 µg/mL. The method is also demonstrated to be robust, as it is resistant to small variations of chromatographic variables such as pH, mobile phase composition, flow rate, and column temperature.

Introduction

Stability is one of the most fundamental aspects of any product's characteristics. Stability testing and forced degradation studies play a very crucial role during drug development to elucidate the intrinsic stability of a drug substance (1). The term "stability indicating assay" has been used to describe "a procedure which affords specific determination of a drug substance in the presence of its degradation products" (2). According to the International Conference on Harmonization (ICH) guideline Q1A (R^2) on the stability testing of new drug substances and products, the stability testing of the drug substance should be carried out under different stress conditions to validate the stability indicating supremacy of the analytical methods used for the analysis of stability samples (3). The prime objective of studying the stability of a drug is to determine the shelf-life of the drug. The identification of the degradation products, the establishment of degradation pathways, the determination of the intrinsic stability of the drug molecules, and validation of the analytical procedure are the goals achieved by stress testing (3). The various conditions specified for the forced degradation studies should include extremes of pH, oxidative and photolytic degradation, and the effect of temperature (4–9). In addition, the need for the stability studies on a drug candidate arises from the fact that the chemical integrity of the drug substance should be maintained until the compound is delivered to the intended site of action. Furthermore, a stability-indicating assay method provides assurance on the detection changes in identity, as well as the purity potency of the product.

Talinolol [1-(4-cyclohexylureidophenoxy)-2-hydroxy-3-tert-butylaminopropane] is categorized as a β1-specific adrenoceptor antagonist (Figure 1), used in the treatment of arterial hypertension, acute and chronic tachycardiac heart arrhythmia, and hyperkinetic heart syndrome (10–12). Talinolol was introduced into clinical practice in 1975 in the former German Democratic Republic (GDR) by AWD (Dresden, Germany), under the trade-

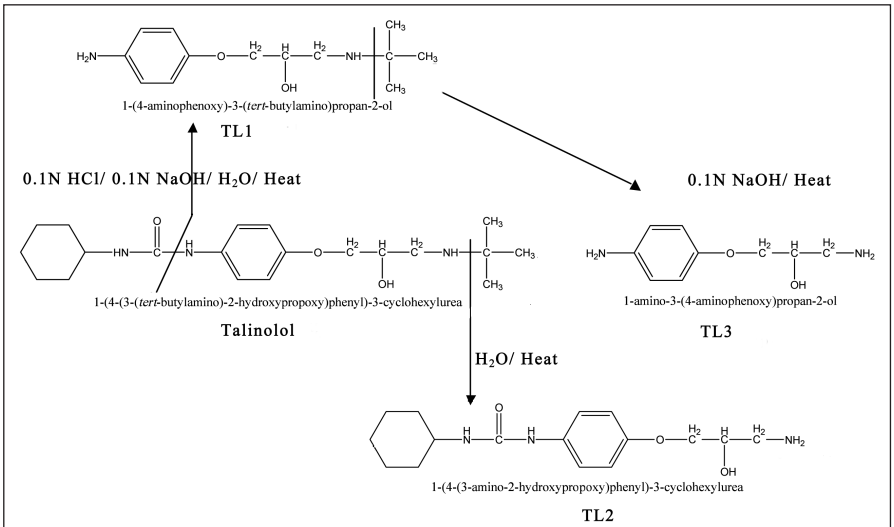

Figure 1. Chemical structures of talinolol and its degraded products with the suggested postulated mechanism for the hydrolytic and thermal degradation of talinolol.

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mark "Cordanum". However, talinolol is not official in any of the pharmacopoeia, but it is available as immediate release tablets with drug contents of 50 and 100 mg, and as a solution for injection in ampoules containing 10 mg talinolol, dissolved in 5 mL of the solvent. Its recommended daily doses range from 25 to 300 mg, in healthy volunteers. Its safety limit was proven up to 400 mg as a single dose (10).

In the literature, various analytical methods have been developed for determination of talinolol in plasma, urine, and in feaces. Oertel et al. used liquid chromatography–tandem mass spectrometry (LC–MS–MS) methods for the determination of talinolol in plasma (13). Talinolol was assayed in serum using a high-performance liquid chromatography (HPLC)–fluorimetric detector method, using 0.025 mol/L triethylammonium phosphate buffer pH 3.0, isocratically mixed with 23% acetronitrile as the mobile phase. A Merck EcoCart 125-3 HPLC cartridge filled with LiChrospher 60 RP-select B column was used at 30°C with a flow rate of 0.8 mL/min (14,15). In another study, talinolol enantiomers were extracted from plasma using a Chiraspher NT stationary phase (250 × 4 mm; Merck) extraction column. The compounds were separated chromatographically with a mobile phase consisting of ethanol–triethylamine in the ratio of 100:0.05 (v/v) (16). While in another method, talinolol was analyzed in plasma, urine, and faeces using XTerra C^{18} MS, 2.1–100 mm, a 3.5 µm pre-column, as well as an XTerra C^{18} MS, 2.1–100 mm, 3.5 µm analytical column attached to a Perkin-Elmer Sciex API 2000 equipped with a mass spectrometer (17). All of these methods were used to quantify talinolol and its metabolites in plasma and excretory products. An improved method for the quantitative determination of talinolol is needed and also required to meet the stability-indicating demand.

Till date, no method has been reported in the literature for the determination of talinolol and also characterization of its degradation products. Therefore, it was decided to carry out the stability study of talinolol towards acidic, alkaline, neutral, thermal, oxidative, and photolytic degradation processes specified as per the ICH guidelines for stress testing. The aim of this work was to develop a stability indicating method for the determination of talinolol and also to characterize its degradation products using reversed phase (RP)-HPLC. Time-of-flight-MS (TOF-MS) with an ESI interface in a positive ion mode and NMR and IR spectroscopy were used for the identification and characterization of the drug and it's degraded products.

Experimental

Materials and reagents

A pharmaceutical grade sample of talinolol (Pioneer Agro Industries, Thane, Maharashtra, India) was used and certified to contain 99.8% of talinolol. The acetonitrile used was of HPLC grade (Merck Ltd., Mumbai, Maharashtra, India). The potassium dihydrogen orthophosphate (S.D. Fine Chem. Ltd., Mumbai, Maharashtra, India), hydrochloric acid (Qualikems, New Delhi, India), and hydrogen peroxide (Qualigens Fine Chemicals, Mumbai, Maharashtra, India) used were of an analytical reagent grade. Reverse osmosis water was used in the study.

Equipment and instrumental conditions

The HPLC (Shimadzu, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 µL loop, and a SPD-10 AVP UV–visible detector. The separation and quantitation were made on a C^{18} reverse phase (250 mm × 4.6 mm, i.d., 5 µm Inertsil ODS-3) column which was operated at 40°C. The detector was set at × 242 nm. The data acquisition was performed on Spinchrom software. Specificity testing was done on Waters (Milford, MA) Delta 600 HPLC equipped with a 600 controller pump, a 2996 PDA detector, and a degasser module. Empower 2 Software was used for data acquisition and processing.

For the investigation of the photostability of talinolol, a stability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with light sources, as defined under option 2 of the ICH guideline Q1B, was used (9). In this chamber, the combination of two black light OSRAM L73 lamps and four white fluorescent OSRAM L20 lamps formed the light bank. The spectral distribution of the black light lamp was between 345 and 410 nm with a maximum at 365 nm, whereas the output of the white fluorescent lamps was similar to that specified in the ISO 10977 (1993). Both the UV and the visible lamps were put on simultaneously. The chamber was maintained at 40°C, and at 75% relative humidity (RH).

Q-TOF Micro (Waters, UK) equipped with an ESI source was used for the identification of the degraded products of talinolol. The TOF-MS analysis worked in a positive ion mode and the mass range was set at *m/z* 50 to 1200. Infusion experiments were carried out to optimize the instrument parameters for maximal generation of protonated molecules and capillary voltage was set to 36 V. Nitrogen gas, set at 50 and 21 psi, was used as a nebulizing and drying gas, respectively. The atmospheric pressure ionization (API) housing and drying gas was kept at 80 and 200°C, respectively. Protonated analyte molecules were subjected to collision, which induced dissociation, using argon as the collision gas at 1.8 mTorr to yield product ions. The capillary voltage and sample cone voltage were set to 2500 V and 30 V, respectively. The pure drug as well as the degraded samples were analyzed exactly at the same MS conditions.

The IR spectrophotometer used was a Perkin-Elmer, Spectrum RX1 FT-IR System (Waltham, MA). The NMR spectra were recorded on a Bruker Avance II 400 NMR spectrometer (200 mHz), Switzerland.

HPLC conditions and standard solutions

The mobile phase was prepared by mixing acetonitrile and potassium dihydrogen orthophosphate buffer (pH 4.4) in a ratio 27:73 (v/v). The flow rate was 1.0 mL/min. All determinations were performed at 40°C. All the samples were analyzed by an HPLC method after filtration using 0.45 µ nylon membrane filters (Millipore, Bangalore, India). The injection volume was 20 µL.

For the preparation of the talinolol standard stock solution (1 mg/mL), accurately weighed 100 mg of talinolol powder was transferred to a 100-mL volumetric flask; to this 50 mL of methanol was added, and the mixture was sonicated for 30 s. The final volume was made up with triple distilled water, and the

resulting solution was vortexed for 1 min. Injections were made in triplicate and chromatographed under the conditions as described herein. The chromatogram of talinolol is depicted in Figure 2.

Validation of method

Linearity

The calibration curve of talinolol was prepared for the establishment of linearity. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph, and the plots were subjected to a linear regression analysis.

Precision

Repeatability studies were performed for the determination of intra-day and inter-day precision. Intra-day precision studies were performed by injecting four different concentrations of talinolol (i.e., 2, 10, 50, and 100 µg/mL) in hexaplicate on the same day. For the inter-day precision studies, these concentrations were injected in hexaplicate on six different days. Drug concentrations were calculated using the area obtained from the linearity plots, and the results are expressed as percent relative standard deviation (RSD).

Limit of quantitation and detection

The solutions of talinolol were prepared at the limit of detection (LOD) and limit of quantitation (LOQ) levels, corresponding to 0.03% (0.125 µg/mL) and 0.1% (0.378 µg/mL), respectively. The LOD and LOQ of the target sample were determined by injecting each concentration in hexaplicate.

System suitability tests

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. The following parameters were used for system suitability evaluation (18,19).

Capacity factor (k'). The capacity factor (the retention factor) is a measure of the retention time of a compound in the sample with a given combination of the mobile phase at a given temperature, and the flow rate in a specified column. It is defined as:

$$k'(A) = (t_A - t_0)/t_0 \qquad \text{Eq. 1}$$

in which t_A is the retention time of the compound, and t_0 refers to retention time for an unretained compound. The t_0 can be calculated by observing the initial baseline deflection of the trace above and below the baseline, caused by the difference in the composition of the sample solutions as well as the mobile phase. An entail baseline deflection of this shape is found to be safe to assure that this corresponds to t_0 (20). In the present work, t_0 was 1.39 min. For an optimum separation, the retention factor should be in the range of $0.5 < k' < 10$.

Selectivity factor (α). The selectivity parameter is a measure of the separation of the two compounds in the sample under the given conditions. For the two components, A and B, it is defined as:

$$\alpha = k'B/k'A \qquad \text{Eq. 2}$$

k' is the respective capacity factor. Therefore, it is the ratio of the relative retentions of the two compounds.

Resolution (R_s). Resolution is a measure of the degree of separation between the adjacent peaks. For the two compounds, X and Y, in a chromatographic run it is expressed as:

$$R_s = 1.18\,(t_{r,Y} - t_{r,X})/(w_{0.5,X} + w_{0.5,Y}) \qquad \text{Eq. 3}$$

in which t_{rX} and t_{rY} are the retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks, and $w_{0.5,X}$ and $w_{0.5,Y}$ are the peak widths at half-height. A value of 1.5 for the resolution implies a complete separation of two compounds (18,19).

Number of theoretical plates (column efficiency). In a particular separation, the number of theoretical plates or column efficiency refers to the performance of the stationary phase, and implies to how well the column was packed. In the present study, the number of theoretical plates was calculated using the following equation:

$$n = 5.54\,(t_R/w_{0.5})^2 \qquad \text{Eq. 4}$$

in which $w_{0.5}$ is the width of the peak at half-height, and t_R is the retention time along the baseline, from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component.

Symmetry factor. The symmetry or tailing factor (T) refers to the peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. The tailing factor of one refers to a symmetric peak. The tailing factor should be calculated using the following equation for chromatographic peaks:

$$T = w_{0.05}/2d \qquad \text{Eq. 5}$$

in which $w_{0.05}$ is the width of the peak at 5% of the peak height,

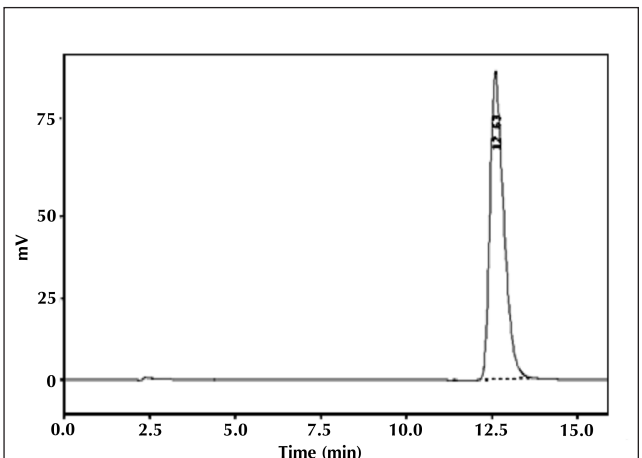

Figure 2. Chromatogram of talinolol. The x-axis and the y-axis of the chromatogram represents the time (min) and the voltage (mV), respectively.

and d is the distance between the perpendicular dropped from the peak maximum, and the leading edge of the peak at one-twentieth of the peak height (18).

Accuracy

The accuracy of the method was assessed by analyzing spiked talinolol samples using independent working standard solutions. Talinolol was spiked into three levels, corresponding to 2, 50, and 100 µg/mL. Additionally, two separate HPLC instruments with different columns on different days were used for the determinations. The percentage recovery of the added drug was then calculated using the linearity plots.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (21). The method specificity and the stability-indicating of the analytical method were evaluated by the ability of the chromatographic conditions to separate the major degradation products from talinolol and, by determination, of the purity of the drug peak in the presence of its degraded sample using a PDA detector. To optimize the HPLC assay parameters, the mobile phase composition was also changed and studied. Peak purity of the talinolol in stressed samples was verified using the PDA in the wavelength range of 200–400 nm.

Robustness

The robustness of the method was established by studying the separation study on a different chromatographic system on a different day. In addition, the experimental conditions were purposely altered, and the resolution between talinolol and its degraded products was evaluated. To study the effect of flow rate and column temperature on the resolution, these were changed by 0.2 units, from 0.8 to 1.2 mL/min, and from 35ºC to 45ºC, respectively. The mobile phase components were held constant as stated in the section: "HPLC conditions and standard solutions".

Stability

Injections of the pure drug and the degraded sample solutions were performed after they were stored at room temperature, protected from light, and also under refrigerated conditions over time in tightly packed containers.

Preparation of the degradation products

Table I shows the different stress conditions used for the forced degradation studies of bulk drug and drug formulations. Zero time samples and placebos (i.e., samples without the drug with other excipients) were also prepared and analyzed for the comparison with the stressed samples. The chromatograms of the blank solutions, consisting of stress agents with and without the drug, and the zero time drug solutions together with the stress agents, were inspected in order to mark the peaks corresponding to the stress agents and to distinguish them from the potential drug degradation products. The stressed samples were also detected under different wavelengths in order to ensure that no additional degradation product(s) were formed with different wavelength values, as compared to talinolol. The total chromatographic run time was 2.5 times more than the retention time of the drug peak.

Acidic conditions

For acidic hydrolysis, hydrochloric acid of different strengths was used for the preparation of 1 mg/mL talinolol solution. 100 mg of talinolol in 100-mL volumetric flask was firstly dissolved in 5 mL methanol, and the volume was made up with 95 mL of 0.01 N HCl, and was kept at 25°C for 2 h, and at 40°C for 8 h. Furthermore, the drug solutions were prepared similarly in 0.1 N HCl and kept at 40°C for 24 h, and also refluxed for 2, 8, and 12 h. The identification of the degradation product was done after the acidic hydrolysis of the drug solution to complete degradation by refluxing for 12 h.

Subsequently, after complete degradation, the pH of the solution was adjusted to precipitate the degraded product of talinolol. The precipitates were filtered, washed, and dried under a vacuum and protected from air and light. The identification and analysis of the degraded products was done by MS-ESI, by injecting the completely degraded drug sample.

Alkaline conditions

Alkaline degradation studies were performed by preparing 1 mg/mL talinolol solution. Talinolol (100 mg) in a 100-mL volumetric flask was firstly dissolved in 5 mL methanol, and the volume was made up to 100 mL with 95 mL of 0.01 N sodium

Table I. Different Stress Conditions for Talinolol (1 mg/mL) with the Percentage Remained after Forced Degradation Studies

Stress	Strength	Conditions exposed	Amount remaining (%)
Acidic	0.01 N HCl	25°C, 2 h	100%
		40°C, 8 h	100%
	0.1N HCl	40°C, 24 h	100%
		2 h reflux	59.10%
		8 h reflux	15.15%
Alkaline	0.01N NaOH	25°C, 2 h	100%
		40°C, 8 h	100%
	0.1N NaOH	40°C, 8 h	100%
		2 h reflux	100%
		8 h reflux	41.50%
Neutral	Water	25°C, 2 h	100%
		40°C, 8 h	100%
		12 h reflux	72.11%
		24 h reflux	15.37%
Thermal	0.01N HCl	70°C, 15 days	66%
	Drug powder	70°C, 15 days	100%
Oxidative	3% H_2O_2	RT, 6 h	100%
		RT, 10 h	100%
		RT, 24 h	100%
	10% H_2O_2	RT, 24 h	100%
	30% H_2O_2	RT, 48 h	99.97%
Light (Wrapped and unwrapped samples)	0.1N HCl	Cool white fluorescent and UV light, 15 days	100%
	Water–methanol		100%
	0.1N NaOH		100%
	Drug powder		100%

hydroxide, and it was kept at 25°C for 2 h and at 40°C for 8 h. Furthermore, the drug solutions that were prepared in 0.1 N NaOH were kept similarly at 25°C for 2 h and at 40°C for 8 h, and also refluxed for 2 h and 8 h.

Neutral (water) conditions

For neutral hydrolysis, 100 mg of talinolol in a 100-mL volumetric flask was firstly dissolved in 5 mL methanol, and the volume made up with 95 mL of water (1 mg/mL), and exposed to different conditions like at 25°C for 2 h and at 40°C for 8 h, and then refluxed for 12 and 24 h.

Thermal degradation studies

To investigate the susceptibility of the drug under thermal stress conditions, the bulk drug was spread in a thin layer in a petri plate, and the drug solution (1 mg/mL in 0.01 N HCl) was exposed to dry heat in a hot air oven at 70°C for 15 days. For the identification, purification, and the analysis of the major degradation products after thermal degradation, the solvent was exposed to freeze drying and protected from air and light. The identification and analysis of the degraded products was done by IR, NMR, and MS-ESI.

Oxidative conditions

Oxidative degradation studies were performed using a hydrogen peroxide solution of different strengths at room temperature. The talinolol solution (1 mg/mL) was prepared in 3% H_2O_2 and exposed for 6, 10, and 24 h. A similar solution was prepared in 10% H_2O_2, and it was analyzed after 24 h. Studies were also carried out on the drug solution in 30% H_2O_2, and kept for 48 h at room temperature.

Photodegradation studies

Photodegradation studies were carried out by exposing the drug solutions (1 mg/mL in 0.1 N HCl, water–methanol mixture, and 0.1 N NaOH) as well as the powder drug in a photostability chamber. The powder was spread as a thin layer in a petri plate. The overall illumination at the point of placement was 6000 lx fluorescent and 0.7 W/m^2 UV light. The samples were withdrawn after 15 days. The samples of both solutions and powder form were kept in parallel in dark (wrapped) for the same period.

Assay of talinolol in tablets and microemulsion formulations

The proposed HPLC method was applied to the determination of talinolol in freshly prepared tablets and microemulsion formulations. Three replicate determinations were made. The contents of 10 indigenously developed talinolol tablets were thoroughly powdered and mixed. An amount of the powder, equivalent to 100 mg of talinolol, was accurately weighed and added to a 100-mL volumetric flask, and 50 mL of methanol was added, and the volume was made up with water. The resulting dispersion was sonicated for 15 min. The dispersion was kept as such for 12 h and vortexed for 5 min, and then filtered through 0.22 µ filters for further analysis.

A similar procedure was followed for the determination of talinolol in stability samples (kept at 40°C/75% RH for three months). Similarly, microemulsion formulations containing talinolol, equivalent to 100 mg, was added to 100-mL volumetric flask, and 50 mL each of methanol and water was added. It was kept as such for 30 min and vortexed for 5 min, and then filtered through 0.22 µ filters for further analysis. Further dilutions of the sample solutions were carried out with water to reach the linearity range specified for talinolol. The general procedures described under calibration were followed, and the concentration of talinolol was calculated.

Results and Discussion

Validation of the method

Linearity

The data obtained from the linearity curve showed that the response of the drug was strictly linear in the studied concentration range from 2 to 100 µg/mL (n = 6). A very high correlation of 0.998 (± 0.0006) was obtained with a slope of 33.51, and with an RSD of 1.13% (Table II).

Precision

The percent RSD value was found to be in the range of 0.19% to 1.6% for intra-day precision studies, whereas the percent RSD values ranged from 0.18% to 1.8% for inter-day precision studies. All data indicates that the method is highly precise for the determination of talinolol (Table III).

Limit of quantitation and limit of detection

For the LOD solution, the mean response of the 6 injections minus 3.3 times the standard deviation (SD) was greater than zero. For the LOQ solution, the mean response of the 6 injec-

Table II. Parameters for the Regression Equation of the Proposed HPLC Method for Talinolol Determination.

Parameters	Talinolol
Calibration range (µg/mL)	2 to 100
Detection limit (µg/mL)	0.125
Quantitation limit (µg/mL)	0.378
Regression equation (Y)*	
Slope (b)	33.51
Standard deviation of the slope (S_b)	0.31
Relative standard deviation of the slope (%)	1.13
Intercept (a)	Zero
Correlation coefficient (r)	0.998

* Y = a + bC, where C is the concentration in µg/mL and Y is the area of the peak.

Table III. Precision Studies

Actual Conc. (µg/mL)	Measured Conc. (µg/mL) ± SD; RSD (%) Repeatability (n = 6)	Intermediate precision (n = 6)
2	2.00 ± 1.20; 1.68	1.94 ± 1.24; 1.80
10	10.15 ± 2.10; 0.60	9.95 ± 0.61; 0.18
50	50.44 ± 3.03; 0.19	49.96 ± 17.84; 1.13
100	101.73 ± 23.17; 0.67	100.65 ± 9.09; 0.26

tions minus 10 times the standard deviation was also found to be greater than zero. Thus, the signal-to-noise levels meet the ICH requirements for LOD and LOQ limits.

Table IV. Results of System Suitability Parameters of the Developed Method for Determination of TL1, TL3, TL2, and Talinolol

Compound	Retention time (min)	Capacity factor (k′)	Selectivity (α)	Resolution factor (Rs)	Theoretical plates (*n*)	Symmetry factor	% RSD of retention time
TL1	2.5	0.78	1.72	4.284	2,404	1.01	0.15
TL3	3.3	1.37	1.47	2.934	13,560	1.14	0.14
TL2	4.2	2.02	3.92	11.884	2,803	1.10	0.26
Talinolol	12.4	7.92			3,362	1.18	0.06

The retention time of unretained peak is 1.39 min.

Table V. Recovery Studies (*n* = 3)

Spiked Conc. (µg/mL)	Measured Conc. (µg/mL) ± S.D; RSD (%)	Recovery (%)
2	2.01 ± 1.70; 2.38	100.5
50	51.18 ± 1.93; 1.24	102.36
100	101.45 ± 3.85; 0.66	101.45

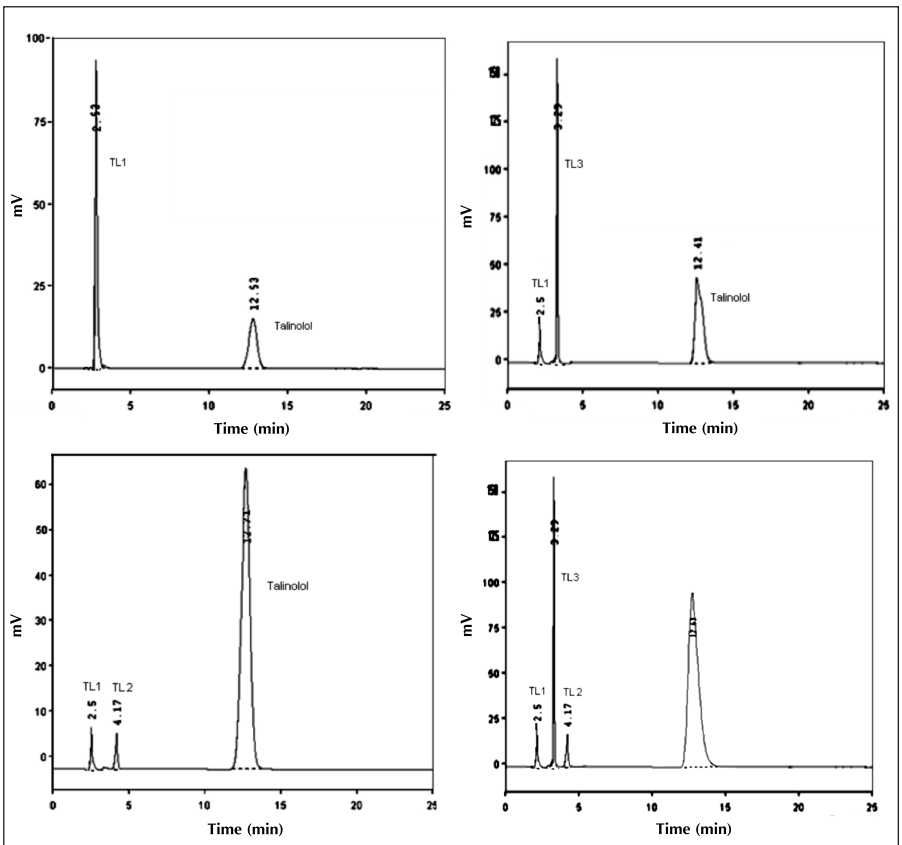

Figure 3. HPLC chromatogram showing the acidic (A), alkaline (B), neutral (C), and thermal (D) degradation of talinolol. The x-axis and the y-axis of each chromatogram represents the time (min) and the voltage (mV), respectively.

System suitability tests

The system suitability tests parameters, such as the capacity factor, the selectivity factor for the separation of each degraded product, the resolution value, and the number of theoretical plates for each compound, are represented in Table IV and were within the desired limits. The calculated value for the tailing factor for each compound (an acceptable range of $0.8 \leq T \leq 1.5$) (18,19) is also represented in Table IV.

Accuracy

The recovery was in the range of 100.5 to 102.36%. The data obtained from the recovery studies is represented in Table V. All data indicated that the method is highly accurate for the determination of talinolol and its degraded samples without any interference.

Specificity

The specificity of the HPLC method is illustrated in Figure 3 where complete separation of talinolol and its degradation products can be noticed. The mixtures of the degraded samples were spiked and the degraded product peaks were found to be well separated from talinolol. A satisfactory separation was obtained by changing the mobile phase composition [i.e., with a mobile phase consisting of a potassium dihydrogen orthophosphate buffer (pH 4.4) and acetonitrile, in the ratio of 73:27 (v/v), at 40°C]. By increasing the acetonitrile concentration to more than 40%, an inadequate separation of talinolol and its degradation products was achieved. At a lower acetonitrile concentration (< 27%), separation occurred, but with excessive tailing for talinolol. The average retention time ± the SD for the degraded products (TL1, TL2, and TL3) and talinolol were found to be 2.5 ± 0.15, 4.2 ± 0.26, 3.3 ± 0.14, and 12.4 ± 0.06 min, respectively, for the six replicates.

In addition, it was also determined that the talinolol peak was spectrally pure, and no degradation product peak was detected as co-elute with talinolol. The studies performed showed the peak purity angle value of 0.050, and the purity threshold value of 0.222. As the purity angle was significantly less than the purity threshold, the method was concluded to be specific for the analysis of talinolol in pharmaceutical formulations.

Robustness

In all the deliberate varied chromatographic conditions (flow rate and column temperature), the resolution between talinolol and its degradation products was not altered, illustrating the robustness of the method (Table VI).

Stability

All solutions were found to be stable for at least 2 months at room temperature. Additionally, degraded sample solutions were found to be stable for 4 months when kept under refrigeration, whereas the pure drug solution was stable for 6 months under refrigeration.

Identification of degraded products

Talinolol was found to be susceptible to hydrolytic and thermal stress (in the solution form). However, it was stable to the oxidative stress, photolytic stress (in both solid and solution form), and the thermal stress (in solid form). Table I shows the summary of the results from forced degradation studies of talinolol, and the percentage of talinolol remained after undergoing stress. The chemical structure of talinolol was studied and shown that there are two preferred sites of hydrolysis: one is a CO–NH– bond, and the another is a NH–$C(CH_3)_3$ bond, which leads to the formation of the degraded products as shown in Figure 1.

The MS-ESI, using selected ion monitoring in the positive ion mode, provided a highly selective method for the determination and characterization of talinolol and its degradation products, respectively. At the optimum conditions, described in the section: "Equipment and Instrumental Conditions", the ESI of talinolol was identified at the mass-to-charge ratio (*m/z*) of 364.4 (corresponding to the $[M+H]^+$ peak), and was the main ion species. Additionally, ion species such as *m/z* 239.2, with the loss of C_6H_{11}–N=C=O from the $[M+H]^+$ ion (i.e., TL1), *m/z* 308.3, with the loss of C_4H_8 from the $[M+H]^+$ ion (i.e., TL2), and *m/z* 183.2, with the loss of C_6H_{11}–N=C=O and C4H8 from the $[M+H]^+$ ion (i.e., TL3), respectively, were present in the respective degraded samples of talinolol. Figure 4 shows the pure drug and degraded product ion mass spectra.

Table VI. Results of Robustness Study.

Parameters	Variations	Resolution between the drug and degradation product peak
Temperature (Flow rate: 1 mL/min)	At 35°C	20.2
	At 45°C	17.5
Flow rate (± 20% of the set flow) at 40°C	At 0.8 mL/min	21.2
	At 1.2 mL/min	17.45

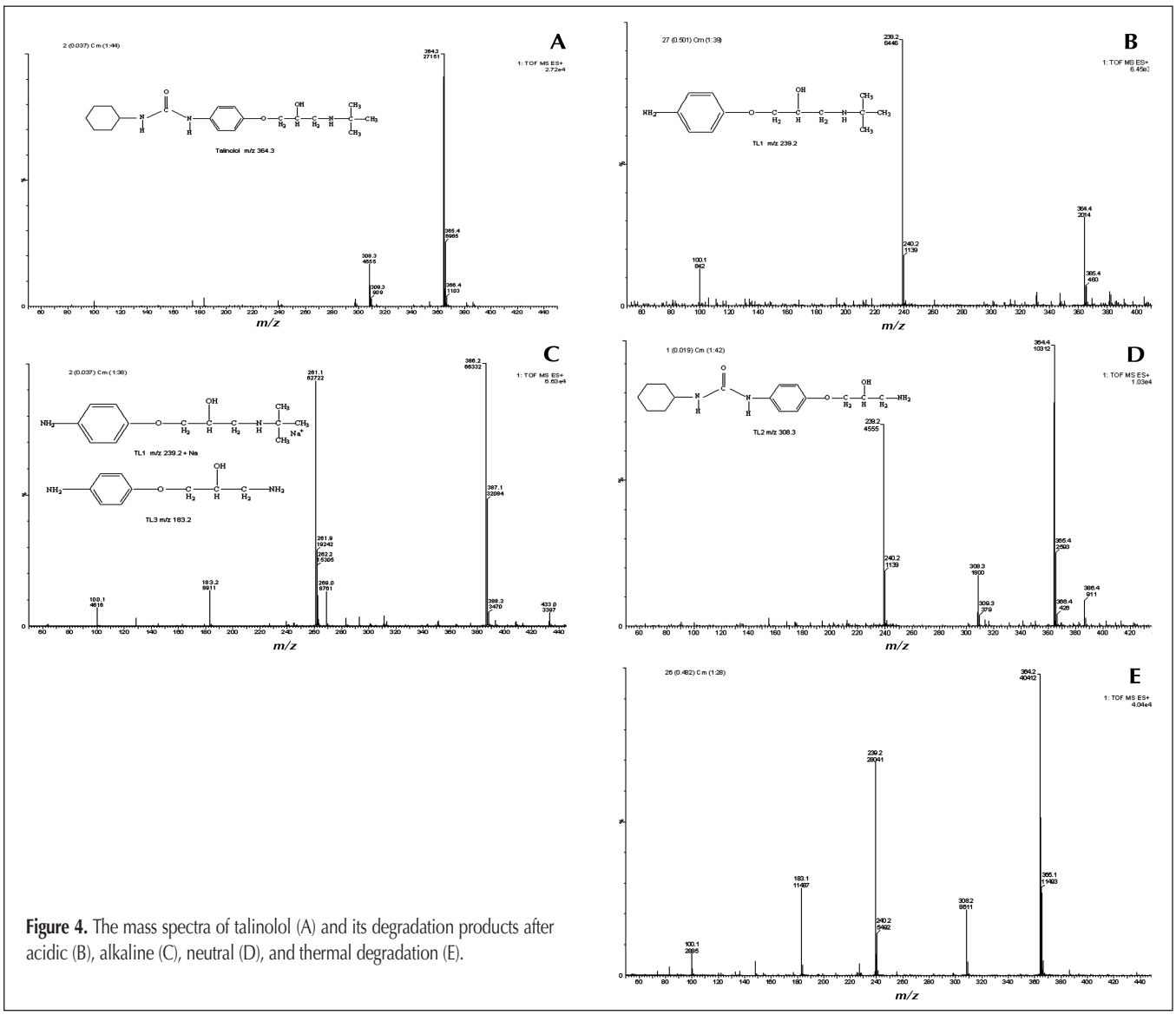

Figure 4. The mass spectra of talinolol (A) and its degradation products after acidic (B), alkaline (C), neutral (D), and thermal degradation (E).

Acidic conditions

The drug was found to be acid labile. The amount of drug remaining after refluxing for 8 h in 0.1 N HCl was just 15.15%. In order to study the effect of a milder condition, refluxing for 2 h in 0.1 N HCl was carried out with 40.90% of drug degradation. The HPLC analysis indicated the presence of a degraded product peak with the reduction in the area of the drug peak (Figure 3A). The peak at the retention time (RT) of 2.53 min was found to be of the major degradation product after refluxing for 2 h, as well as for 8 h in 0.1 N HCl. The shorter retention time of the degraded product indicates a different polarity or a lower molecular mass, as compared to talinolol. For further milder conditions, the drug showed no degradation.

When talinolol was refluxed with 0.1 N of hydrochloric acid for 12 h, the degradation product of talinolol could be isolated and identified from the reaction mixture. The assignments of the dried precipitates of the degradation product as TL1 (Figure 1) was based on the comparison of the IR, NMR, and the mass spectral (*m/z* 239.2) data of intact talinolol with those of the purified degraded sample and separated from the degradation reaction.

The IR spectrum (KBr) of talinolol was characterized by the absorption frequency of C=O carbonyl of the amide band at 1626 cm^{-1}. On the other hand, the IR spectrum (KBr) of TL1 lacked the characteristic carbonyl band (Figure 5).

The NMR spectrum of talinolol in DMSO-$2d_6$ was characterized by the appearance of the protons of the methyl groups at δ 1.10 ppm (singlet, 9H, CH_3), the protons of the cyclohexane at δ 1.39–1.78 ppm (doublets, 10H, CH_2), protons of urea; aromatic N*H* at δ 5.5 ppm (singlet, N*H*), and another N*H* at δ 6.9 ppm (broad singlet, N*H*), and the proton of cyclohexane, which is under influence of N–C=O at δ 3.54 ppm (doublet, C*H*). The alcohol and the amine protons were at δ 2.0 ppm (doublet, 2*H*, N*H*, and O*H*). The aromatic CH protons were at δ 6.75 ppm (doublet, 2*H*, C*H*), and at δ 7.08 ppm (doublet, 2*H*, C*H*). By contrast, the NMR spectrum of TL1 in the same solvent lacked the characteristic cyclohexane and urea protons signals, and showed additional protons of aromatic NH_2 at δ 4.0 ppm (doublet, 2*H*, C–N*H*). The spectrum also showed the protons of the methyl groups were at δ 1.10 ppm (singlet, 9*H*, CH_3), and the alcohol and amine protons were at δ 2.0 ppm (doublet, 2*H*, N*H*, and O*H*). The IR and NMR spectrum indicates that TL1 lacks the C=O carbonyl and cyclohexane ring with urea moiety of talinolol, respectively.

Furthermore, the MS spectra of talinolol (Figure 4A), the most abundant fragmentation ions of talinolol, at *m/z* 364.4 ($[M+H]^+$), was selected for quantification. Whereas in Figure 4B, *m/z* 239.2 was the most abundant ion, with a sufficient reduction in intensity of *m/z* 364.4 $[M+H]^+$, showing the formation of a degraded product. This was previously supported with the LC–UV chromatogram of the stressed talinolol (Figure 3A). Fragment ions species such as *m/z* 100.1 (corresponding to $[C_6H_{11}–NH_2+H]^+$ ion from $[M+H]^+$) occurred in a minute amount.

The acidic hydrolysis of CO–NH– bond would normally be the favored process leads to the formation of TL1 (Figure 1). After thorough comparison and interpretation of the LC–UV, IR, NMR, and the mass spectra of both the talinolol and of TL1, it can be concluded that the degradation product (TL1) eluted at about 2.53 min and gave the molecular ion peak at *m/z* ratio 239.2, while the molecular ion peak of talinolol was at *m/z* ratio 364.4 and elutes at 12.5 min.

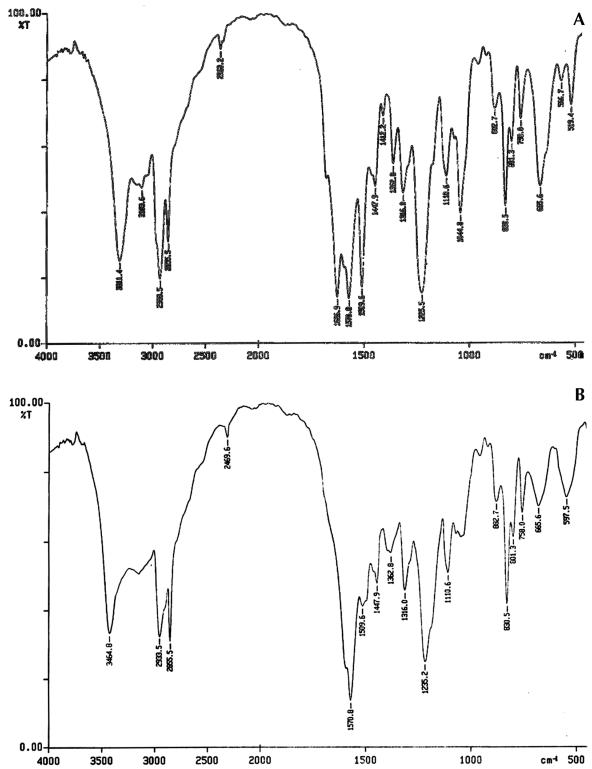

Figure 5. The IR spectra of talinolol (A) and TL1 (B).

Alkaline conditions

The drug was found to be more stable to alkaline hydrolysis as compared to acidic hydrolysis. The drug solution kept at 25°C for 2 h, at 40°C for 8 h in 0.01 N NaOH, and at 40°C for 8 h in 0.1 N NaOH showed no degradation. The drug solution kept for reflux for 2 h in 0.1 N NaOH showed no degradation, but when the reflux was continued for 8 h, 58.50% of the drug got degraded. The HPLC analysis indicated the presence of the two degraded products with the occurrence of two peaks (Figure 3B), along with decrease in the area of drug peak. The degradation product peaks arises at RT 2.5 and 3.29 min, respectively. As compared to acidic hydrolysis, one additional peak arises at 3.29 min, indicating the formation of another chromophoric compound.

When refluxed with 0.1 N NaOH for 8 h, TL1 and TL3 could be identified as a degradation product of talinolol, which was difficult to be isolated from the reaction mixture. The MS data depicted the formation of TL1 and 1-amino-3-(4-aminophenoxy) propane-2-ol, *m/z* 183.2, (i.e., TL3) as the degradation products of talinolol in an alkaline medium (Figure 1). The assignments of the degradation products, TL1 and TL3, was based upon the comparison study of the data obtained from the acid degradation as well as the mass spectra of the alkaline degraded sample (Figure 4C), with that of talinolol.

The MS spectra of the alkaline degraded sample shows two ion fragment species, in addition to *m/z* 386.2 $[M+Na]^+$. The peak at *m/z* 261.1 (with the loss of C_6H_{11}–N=C=O from the $[M+Na]^+$ ion) and at *m/z* 183.2 were the most abundant ion fragments, with a

sufficient reduction in intensity of *m/z* 364.4 $[M+H]^+$. It was also previously concluded with an LC–UV chromatogram of the alkaline stressed sample of talinolol. The MS of TL1 in the presence of a Na^+ ion gives the ion fragment spectra at *m/z* 261.1, which is corresponding to *m/z* 239.2 and identified as TL1, as in the section: "Validation of the method: Acidic conditions".

The breakdown of TL1 at the NH–$C(CH_3)_3$ bond could lead to the formation of TL3 (Figure 1). In conclusion, the LC–UV and the MS-ESI study confirm that the degradation product, which eluted at about 2.5 and 3.29 min, gave the molecular ion at *m/z* ratio 261.1 and 183.2 (corresponding to TL1 and TL3, respectively), while the molecular ion of talinolol was at *m/z* ratio 364.4 and elutes at 12.4 min.

Neutral conditions

The drug underwent neutral hydrolysis, and a sufficient degradation of 27.89% and 89.63% was observed after a reflux of 12 and 24 h, respectively. The drug solution kept at 25°C for 2 h and at 40°C for 8 h showed no degradation. The LC–UV analysis of the 24 h degraded sample showed that the drug degraded to two major degradation products with a relative retention time (RRT) of 2.5 and 4.17 min, respectively (Figure 3C). The area of the drug peak was also found to be decreased. The peak at an RT of 4.17 min reveals to the formation of a new chromophoric degraded product, which was difficult to be isolated from the reaction mixture. This was identified as 1-[4-(3-amino-2-hydroxypropoxy) phenyl]-3-cyclohexylurea, *m/z* 308.3 (i.e., TL2). The MS spectra of the neutral degraded sample shows two ion fragment species, that are *m/z* 239.2 and *m/z* 308.3, as shown in Figure 4D.

The breakdown of talinolol at the CO–NH– bond and at the NH–$C(CH_3)_3$ bond could normally be favored process leading to the formation of TL1 and TL2, respectively (Figure 1).

Thermal degradation studies

Talinolol was stable in a solid state under thermal stress conditions. However, when a solution of the drug was kept at 70°C for 15 days, 34% of the drug degraded. The HPLC analysis indicated the reduction in the drug peak area with the occurrence of three new peaks, with an RRT of 2.5, 3.3, and 4.17 (Figure 3D). The NMR spectrum of the purified freeze dried precipitates in DMSO-d_6, characterized as TL2; it lacked the protons of the methyl groups at δ 1.10 ppm (singlet, 9*H*, CH_3), as compared to the NMR spectra of talinolol, and showed a shift in proton of cyclohexane at δ 1.44–1.66 ppm (doublet, 5*H*, CH_2). The spectra also showed the protons of urea; aromatic N*H* at δ 5.5 ppm (doublet, N*H*), and another N*H* at δ 6.9 ppm (broad singlet, N*H*), and a proton of cyclohexane, which is under the influence of N–C=O from methane at δ 3.54 ppm (doublet, C*H*). The alcohol and the amine protons were at δ 2.0 ppm (doublet, 2*H*, N*H*, and O*H*). The aromatic C*H* protons were at δ 6.75 ppm (doublet, 2*H*, C*H*) and δ 7.08 ppm (doublet, 2*H*, C*H*).

The mass spectrum of the thermal degraded sample is depicted in Figure 4E. The results of the hydrolytic degradation in combination with the LC–UV and MS-ESI study helps to conclude that the peaks at 2.5, 3.3, and 4.17 min correspond to TL1, TL3, and TL2, respectively, as interpreted in the sections "Validation of the method: Acidic conditions" to "Validation of the method: Neutral conditions". The breakdown of TL1 at the NH–$C(CH_3)_3$ bond could normally be favored process, which leads to the formation of TL3 (Figure 1).

Oxidative conditions

The drug was found to be stable to the oxidative stress without any reduction in the drug peak area. No degradation of the drug was observed in 3% H_2O_2 on exposing initially for 30 min and then for 3 h in 10% H_2O_2 for 6 and 24 h, and in 30% H_2O_2 for 24 h and then for 48 h. Very little degradation was observed on exposing to 30% H_2O_2 for 48 h (Table I).

Photodegradation studies

Talinolol was found to be stable in all solution forms as well as in solid state to light, both in wrapped and unwrapped samples after the exposure equivalent to ICH dose of light.

Assay of Pharmaceutical formulations of talinolol

A typical chromatogram obtained for the quantitative analysis of talinolol in freshly prepared pills was very similar to that presented in Figure 2. The amount of talinolol in tablets was initially found to be 99.31 ± 0.98%, and when kept for three months at 40°C/75%, RH was 97.24 ± 1.3% with an RSD of 0.58% and 1.63%, respectively. The amount of talinolol in microemulsions was found to be 99.50 ± 1.7% (initially) and 94.36 ± 0.26% (After three months at 40°C/75 RH) with an RSD of 1.027% and 1.26%, respectively. This also showed that the method is simple and sensitive for the determination of talinolol in pharmaceutical formulations.

Conclusion

The present study was conducted to develop and validate a simple, sensitive, and reproducible stability-indicating isocratic RP-HPLC method for the quantitative determination of talinolol with the characterization of its degradation products. The identification of degraded products as TL1, TL2, and TL3 of talinolol was a challenging task. Talinolol was found to be labile to neutral and alkaline hydrolysis, and was highly labile to acidic hydrolysis. The drug was also labile to heat when exposed in solution form. However, it was stable to the oxidative stress, photolytic stress, and to the thermal stress (in solid form). The developed chromatographic assay fulfilled all the requirements to be identified as simple, specific, selective, reliable and feasible method, including accuracy, linearity, recovery, and precision data.

The data generated from the performed forced degradation studies enabled the evaluation of talinolol stability under a variety of ICH recommended conditions. Such data is valuable for the safety and potency assessment of a drug product. This study also brings forward new and interesting aspects on the stability of talinolol, as no such systematic study is known earlier in the literature. Furthermore, this simple and rapid isocratic RP-HPLC method can also be used successfully for the determination of talinolol in pharmaceutical formulations without any interference from the excipients. So, the proposed chromatographic procedure confirmed its applicability as a stability-indicating method.

Acknowledgments

The authors are obliged to the Indian Council of Medical Research (ICMR) (New Delhi, India) for providing financial support through a senior research fellowship for this project. The authors are highly thankful to department of CIL, Panjab University, Chandigarh, for providing the use of MS and NMR equipment.

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Manuscript received June 2, 2010;
revision received August 20, 2010.