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A Simple Stability Indicating Method Development and Validation for the Simultaneous Estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride in Pharmaceutical Dosage Forms by RP-HPLC

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ABSTRACT

A reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for simultaneous estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride in pure and marketed formulations. Separation was carried out using column Hypersil ODS C18 (250 mm x 4.6mm x 5µm particle size) in isocratic mode using mobile phase composition pH 6.0 ammonium acetate Buffer: Acetonitrile (68:32)v/v and UV detection at 310 nm. The compounds were eluted at a flow rate of 1.0 mL/ min. The average retention times for Naloxone and Buprenorphine were 2.86 and 3.67 min, respectively. The method was validated according to the ICH guidelines. The %RSD of all validation parameters found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. The method was linear over the concentration of 5-30µg/ml and 20- 120µg/ml for Naloxone and Buprenorphine respectively. The LOD and LOQ of Naloxone were found to be 0.08µg/mL and 0.26µg/mL and of Buprenorphine were found to be 0.0078µg/mL and 0.0237µg/mL. The drugs were also exposed to acidic, alkaline, oxidative, thermal and photolytic conditions and the stressed samples were analyzed by the proposed method. Degradation studies showed that the both the drugs were highly stable under acidic, oxidative, thermal and photolytic conditions. Under alkaline conditions, RT values were shifted to lower as compared to standard without any additional peaks. The high percentage of stability under stress conditions confirms the suitability of the method for simultaneous estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride in pure and marketed formulations.

INTRODUCTION

Naloxone hydrochloride (NAH) is chemically known as Morphinan-6-one, 4, 5-epoxy-3, 14-dihydroxy-17-(2-propenyl), Hydrochloride. The empirical formula of NAH is $C_{19}H_{22}ClNO_4$ and the molecular weight is 363.835 (Fig. I). It occurs as a white to slightly off-white powder, and is soluble in water, in dilute acids, and in strong alkali; slightly soluble in alcohol, insoluble in ether and in chloroform. It is a potent opioid antagonist and is a competitive antagonist at mu, delta and kappa opioid receptors. Naloxone is synthesized from thebaine. The chemical structure of naloxone resembles that of oxymorphone, the only difference being the substitution of the *N*-methyl group with an allyl (prop-2-enyl) group. The name naloxone has been derived from *N*-allyl and oxymorphone[1-4].

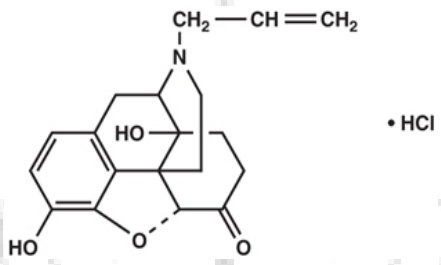


Fig: I Structure of Naloxone Hydrochloride

Buprenorphine hydrochloride (BUH) is chemically known as (6R, 7R, 14S)-17cyclopropylmethyl-7, 8-dihydro-7-[(l S)-1-hydroxy-1, 2, 2trimethylpropyl]-6-0-methyl-6, 14-ethano-17-normorphine hydrochloride. The molecular formula of BUH is $C_{29}H_{42}ClNO_4$ and the molecular weight is 504.1 (Fig.II). Buprenorphine hydrochloride is a white or off-white crystalline powder, weakly acidic and sparingly soluble in water, freely soluble in alcohols and practically insoluble in cyclohexane. It is a potent semi-synthetic opiate analgesic with a potency of 20-40 times higher than that of morphine [5]. BUH is a clear, sterile, injectable agonist-antagonist analgesic intended for intravenous or intramuscular administration successfully or sublingual routes for the treatment of moderate to severe pain as well as chronic pain [6]. Thus BUH is given in combination with antagonist NAH. A few examples of combinatorial dosage forms of BUH and NAH currently available in the market in different dosage forms by their trade names are SUBOXONE [7].

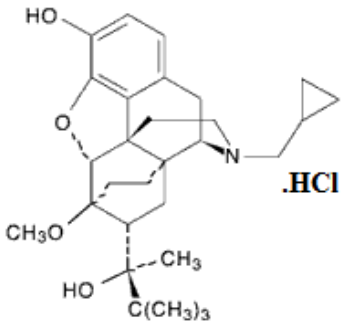


Fig: II Structure of Buprenorphine Hydrochloride

Literature survey reveals that there were number of analytical methods available for both the drugs alone or in combination with other drugs including spectroscopy, chromatographic methods such as gas chromatography with electron-capture or mass spectrometry detection and HPLC with fluorescence electrochemical or mass spectrometry detection [8-13] are reported, but there is no method established for the stability indicating RP-HPLC under stress for this combination. The present work describes the development of stability indicating RP-HPLC method, which can quantify these components simultaneously from a combined dosage form. The present RP-HPLC method was validated [14-15] and applied under stressed conditions according to (ICH) guidelines. ICH has made the mandatory need of developing stability indicating assay methods for every drug candidates. Stability indicating assay methods helps in establishing the inherent stability of the drug which provides assurance on detection changes in identity, purity and potency of the product on exposure to various conditions [16]. So an attempt has been made to develop a method under stress conditions like acidic, basic, thermal, photolytic and oxidative, this which in turn can help in establishing the degradation pathways and the intrinsic stability of the molecules. The object of the present work was to develop a stability indicating method for the simultaneous estimation of Naloxone hydrochloride and Buprenorphine hydrochloride.

MATERIALS AND METHODS

EXPERIMENTAL

Instruments and columns

Shimadzu with high pressure liquid chromatographic instrument provided with a LC 20 AD Pump and Prominence SPD 20A UV-deuterium detector. Data acquisition was performed by using Spin chrome software, Shimadzu Class VP version 6.12 SPS data system. Power Sonicator, model no: 405, Hwashin Technology, Korea. The column used in the development for determination is Hypersil ODS C_{18} (250 mm x 4.6 mm; 5µm).

Chemicals used

HPLC grade Acetonitrile, methanol and water were purchased from E.Merck Co; Mumbai, India and Ammonium acetate, glacial acetic acid AR grade were purchased from SD Fine Chem. Mumbai, India. The reference samples of Buprenorphine Hydrochloride and Naloxone Hydrochloride were supplied by Spectrum Analytical Labs, Hyderabad, Telangana State, India, and branded formulation was purchased from local market.

Chromatographic conditions:

Table : I Chromatographic Conditions

Method parameters	Optimisation conditions
Column	Hypersil ODS C18, 250 X 4.6 mm, 5µm,
Flow Rate	1.0 ml/min
Wavelength	310 nm
Column température	30°C
Injection volume	10 µL
Run time	6 minutes
Mobile phase	pH 6.0 ammonium acetate buffer: Acetonitrile (68:32)v/v
Elution	Isocratic
Needle wash	Water: Acetonitrile 90:10 (v/v)

Selection of wavelength (λ_{max})

An ideal wavelength is one that uses good response for the drugs to be detected Buprenorphine Hydrochloride and Naloxone Hydrochloride in diluents the spectra was scanned on UV- visible spectrophotometer in the range of 200 nm to 400 nm against diluents as blank. The maximum absorbance of both drugs was found to be at 310 nm. So the 310 nm was selected for simultaneous estimation Buprenorphine Hydrochloride and Naloxone Hydrochloride in Pharmaceutical Dosage Forms.

Preparation of mobile phase

Weighed accurately 770mg of ammonium acetate and dissolved in 100ml of water and volume was made up to 1000 mL with water adjust the PH to 6.0 using glacial acetic acid. The solution was filtered through 0.45µ membrane filter and was degassed. A freshly prepared binary mixture of Ammonium acetate and glacial acetic acid buffer: Acetonitrile in a ratio of (68:32) V/V was used as the mobile phase. Methanol was used as diluents for preparing the working solution of the drug. The mobile phase was filtered through 0.05µ membrane filter and sonicated. The flow rate of the mobile phase was maintained at 1.0 mL/min. The column temperature was maintained at 30ºC and the detection of the drug was carried out at 310 nm.

Preparation of stock solution

Weighed accurately about 5mg of Naloxone Hydrochloride and 20mg Buprenorphine Hydrochloride transferred into 25 mL volumetric flask. The solution was sonicated and filtered through Whatman filter paper; resulting solution was diluted with the mobile phase to get a working standard solution.1mL from the above Stock solutions of Naloxone Hydrochloride and Buprenorphine Hydrochloride was taken into a 10 mL volumetric flask and diluting up to the mark with the mobile phase. Mixed standard solutions of different concentrations ranging from 5 – 30µg/mL of Naloxone Hydrochloride and 20 – 120µg/mL of Buprenorphine Hydrochloride were prepared by taking suitable aliquots of working standard solution in different 10 mL volumetric flasks and diluting up to the mark with the mobile phase.

Preparation of sample solution

Twenty tablets were weighed and average weight was determined and finally powdered. Tablet powder equivalent to 0.5 mg Naloxone Hydrochloride and 2 mg Buprenorphine Hydrochloride was accurately weighed and transfer to 10 mL volumetric flask. The contents were sonicated for about 15 min for complete solubility of the drug after adding 10 mL of mobile phase and the volume was made up to the mark with mobile phase. Then the mixture was filtered through a 0.45µ membrane filter. From the above solution, 4 mL aliquot was taken into a separate 10 mL volumetric flask and diluted up to the volume with the mobile phase and mixed well.

Optimization of HPLC method

The HPLC method was optimized with an aim to develop an accurate and precise method for the estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride in pharmaceutical dosage forms. For the method optimization different mobile phases were tried but acceptable retention times, theoretical plates and good resolution observed with pH 6.0 ammonium acetate buffer: Acetonitrile in a ratio of (68:32) v/v was used as the mobile phase using Hypersil ODS C18, 250 X 4.6 mm, 5µm.

Validated RP-HPLC Method for Naloxone Hydrochloride and Buprenorphine Hydrochloride

Validation of the optimized method was performed according to the ICH guidelines **[14-15].**

Linearity:

A linear relationship was evaluated across the range of the analytical procedure with a minimum of six concentrations. A series of standard dilutions of NAH and BUH were prepared over a concentration range of 5-30µg/mL and 20-120µg/mL from stock solution and injected. Linearity is evaluated by a plot of peak areas as a function of analyte concentration, and the results were evaluated by using the statistical methods like slope, intercept, and regression (R^2) correlation coefficients (R) and the data was given in table-II-IV (Fig.III,IV).

Table: II Linearity Data for Naloxone Hydrochloride

S.NO	Concentration of Naloxone (µg/mL)	Peak Area (μV^2 Sec)
1	5	223185
2	10	451147
3	15	641147
4	20	864105
5	25	1110943
6	30	1313835

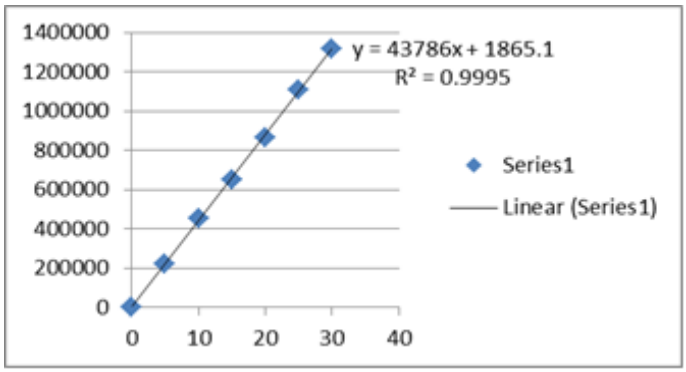


Fig III: Calibration curve for Naloxone Hydrochloride

Table III: Linearity Data for Buprenorphine Hydrochloride

S.NO	Concentration of Buprenorphine (µg/mL)	Peak Area (μV^2 Sec)
1	20	777071
2	40	1594484
3	60	2462354
4	80	3246452
5	100	4051593
6	120	4779266

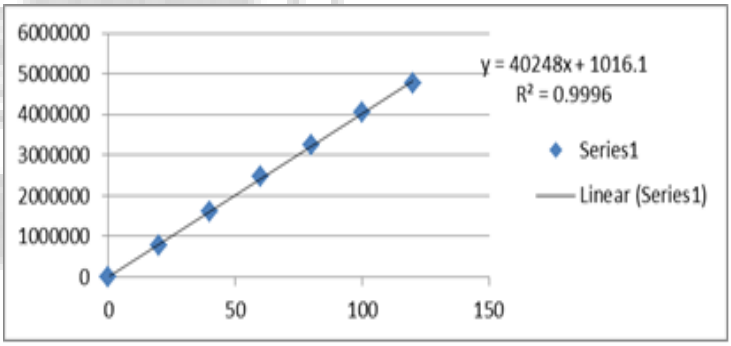


Fig IV: Calibration curve for Buprenorphine Hydrochloride

Precision

Repeatability expresses the precision under the same operating conditions over a short Interval of time. The six repeated homogenous injections of standard solutions were made about 20µg/mL Naloxone and Buprenorphine 80µg/mL and the response factor of drug peaks, mean, standard deviation and % RSD were calculated. Repeatability data for NAH and BHU are summarized in (table IV).

Method Precision

Method precision was determined by injecting six sample solutions of Single batch were analysed as per test method. The mean, standard deviation and % RSD for peak areas of Naloxone and Buprenorphine from sample solutions were calculated. The results were given in the table-IV.

Accuracy

For accuracy determination, three different concentrations were prepared separately i.e.50%, 100%, and 150% of analyte and the chromatograms were recorded for the same. The results obtained for recovery were found to be within the limits. The results were given in the table-V.

Table: IV Summary of Validation Parameters

S.No	Validation Parameter	NAH	BUH
1	Detection of wavelength	310nm	
2	Linearity Calibration range (µg/ml)	5-30	20-120
3	Regression Equation Y=mX+C	Y=43786x+1865.1	Y=40248x1016.1
4	Slope (m)	43786	40248
5	Intercept (C)	1865	1016
6	Correlation coefficient (r)	0.9995	0.9996
7	System Precision (n=6) %RSD	0.72	0.7
8	Method precision (n=6) %RSD	0.03	0.08
9	Specificity Interference from mobile phase, diluents, placebo and degradants	no interference at the retention time of both drug peaks	
10	LOD(µg/mL)	0.08	0.26
11	LOQ(µg/mL)	0.0078	0.0237

Table: V Results for Accuracy

Accuracy	Amount added(µg/ml)		Amount recovered(µg/ml)		Percentage recovered	
	NAH	BUH	NAH	BUH	NAH	BUH
50%	10	40	10.06	40.04	100.61	100.09
100%	20	80	20.13	80.82	101.57	100.02
150%	30	120	30.26	121.66	100.81	101.36
Overall mean of three levels % recovery					101.74	

Robustness

To evaluate the robustness, the following small deliberate variations are made in the method and analyzed the sample in triplicate. 1.Column oven temperature (±5^{0}C), 2.Flow rate (±10%), 3.change in buffer composition (±5%).

The system suitability was evaluated in each condition and compared with the results of method precision. The results were given in the Table-VI.

Table: VI Robustness data for Naloxone and Buprenorphine.

S No	Name	Naloxone		Buprenorphine	
		RT (min)	Area (µV^2 Sec)	RT (min)	Area (µV^2 Sec)
1.	Robustness-1 flow rate at 0.9 mL/min	2.851	926975	3.655	3534030
2.	Robustness-2 flow rate at 1.1mL/min	2.848	920578	3.659	3513327
3.	Robustness-3 Column oven temperature at 25^{0}C	2.811	886532	3.542	3449078
4.	Robustness-4 Column oven temperature at 35^{0}C	2.809	886632	3.536	3448564
5.	Robustness-5 Buffer variation at 63:37	2.837	923619	3.634	3472098
6.	Robustness-6 Buffer variation at 73:27	2.838	923140	3.635	3499035

Specificity

Specificity shall be established by demonstrating that the procedure is unaffected by the presence of interference at the retention time of the Naloxone Hydrochloride and Buprenorphine Hydrochloride with respect to mobile phase, Diluents, placebo and degradants. The specificity studies include deliberate degradation of the tablet sample by exposure to stress conditions, Specificity studies also include blank, placebo solution, and sample solution (control sample), Naloxone and Buprenorphine standard solution were injected into the HPLC system. There was no interference from the blank and placebo at the retention time of the peaks. Peak purity data reveals that Naloxone and Buprenorphine were homogeneous and there was no interference at the retention time of both drug peaks (Fig.V).

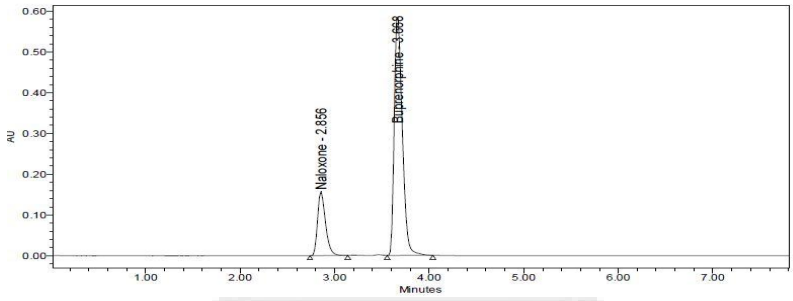


Fig: V Standard Chromatogram of NAH and BUH

Degradation Studies:

Forced degradation or stress studies are undertaken to demonstrate specificity. The objective of developing stability- indicating methods were particularly little information is available about potential degradation products. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development manufacturing and packaging in which knowledge of chemical behavior can be used improve a drug product.

Forced Degradation study was carried out by treating the sample under the following conditions [16]. Twenty tablets were weighed and average weight was determined and finally powdered. Tablet powder equivalent to 0.5 mg Naloxone Hydrochloride and 2 mg Buprenorphine Hydrochloride was accurately weighed and transfer to 10 mL volumetric flask. The contents

were sonicated for about 15 min for complete solubility of the drug after adding 10 mL of mobile phase and the volume was made up to the mark with mobile phase. Then the mixture was filtered through a 0.45µ membrane filter. From the above solution, 4 mL aliquot was taken into a separate 10 mL volumetric flask and diluted up to the volume with the mobile phase and mixed well.

Acid Degradation

To 1 ml of stock solution of NAH and BUH, 1ml of 2N Hydrochloric acid was added and refluxed for 2 hrs at 60^0C.The resultant solution was diluted to obtain 20µg/ml & 80µg/ml solution and10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig:VI).

Alkali Degradation

To 1 ml of stock solution Naloxone and Buprenorphine, 1 ml of 2N sodium hydroxide was added and refluxed for 2hrs at 60^0c. The resultant solution was diluted to obtain 20µg/ml & 80µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample (VII).

Thermal Degradation

The standard drug solution was placed in oven at 105°C for 6 hrs to study thermal degradation. For HPLC study, the resultant solution was diluted to 20µg/ml & 80µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample (VIII)**.**

Peroxide Degradation

To 1 ml of stock solution of Naloxone and Buprenorphine, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 1 hr at 60^0c. For HPLC study, the resultant solution was diluted to obtain 20µg/ml & 80µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig.IX).

Photo Stability:

The photochemical stability of the drug was also studied by exposing the 20µg/ml & 80µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7 days or 200 Watt hours/m^2 in photostability chamber. For HPLC study, the resultant solution was diluted and 10µl were injected into the system under stabilized chromatographic conditions (Fig.X).

Table: VII Forced degradation data for NAH and BUH

Condition	Retention time (min)		Area(μV^2 Sec)		% of Active drug Present after Degradation	
	NLX	BNP	NLX	BNP	NLX	BNP
Control sample	2.848	3.656	919827	3513384	20.75	79.25
Acid Degradation	2.863	3.675	857726	3294421	21.77	75.99
Alkaline Degradation	2.341	2.732	865469	3345775	18.84	80.99
Thermal Degradation	2.849	3.660	872467	3385878	20.79	79.21
Peroxide degradation	2.848	3.659	841906	3210431	20.74	78.99
Photolytic degradation	2.848	3.653	887197	3399592	20.79	79.21

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the developed method were determined by analysing progressively low concentration of the standard solutions using the developed methods. The results are given in the table-IV

$$LOD = 3.3\ \sigma / S \text{ and } LOQ = 10\ \sigma / S$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

Analysis of marketed formulations

The fixed chromatographic conditions were applied for the estimation of Naloxone and Buprenorphine (0.5 mg of Naloxone and 2 mg of Buprenorphine) formulation by RP-HPLC method. Twenty tablets were weighed and average weight was determined and finally powdered. Tablet powder equivalent to 0.5 mg Naloxone Hydrochloride and 2 mg Buprenorphine

Hydrochloride was accurately weighed and transfer to 10 mL volumetric flask. The contents were sonicated for about 15 min for complete solubility of the drug after adding 10 mL of mobile phase and the volume was made up to the mark with mobile phase. Then the mixture was filtered through a 0.45µ membrane filter. From the above solution 4 mL aliquot was taken into a separate 10 mL volumetric flask and diluted up to the volume with the mobile phase and mixed well. Initially, inject 20µL of blank solution, placebo solution, sample solution and standard solution, Disregard peaks due to blank and placebo if any. The results were given in the table-VIII

Table: VIII Analysis of marketed formulations (Assay) data for NAH and BUH

Drug	Quantity claim (mg/tablet)	*Quantity found (mg/tablet) ± SD	*% Assay found ± SD
Naloxone	**0.5**	**0.50 ±0.009**	**100.05 ± 0.60**
Buprenorphine	**2**	**2.03 ± 0.008**	**101.56± 0.92**

Recording of chromatograms

The standard solutions stabilize the system until stable obtained. Initially, inject the blank solution and placebo. The standard chromatograms were recorded by injected standard solutions and the peak areas of standard chromatograms were noted. Calibration graph was plotted using peak area versus concentration. Then the sample solution was injected and the amount of Naloxone and Buprenorphine present in the formulation was calculated from the calibration curve. The amount of Naloxone and Buprenorphine present in per tablet Naloxone and Buprenorphine was found to be 0.50 ± 0.009mg and 2.03 ± 0.008mg. Total label claim for (0.5mg of Naloxone and 2mg Buprenorphine) formulation (Fig.XI).

RESULTS AND DISCUSSION

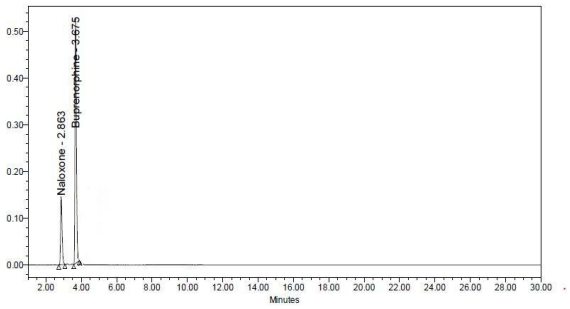
The goal of the study is to development of simple, rapid, sensitive, specific and accurate HPLC methods for the routine quantitative determination of samples. Hypersil C_{18} ODS Column (250 mm x 4.6 mm; 5µm) as stationary phase. The mobile phase composition ammonium acetate and glacial acetic acid buffer: Acetonitrile in the ratio of 68:32 and pH adjusted to 6.0±0.1 with glacial acetic acid selected. A good linear relationship (r^2 = 0.9995 & r^2 = 0.9996) was observed

in the range of 5-30 µg/mL & 20µg/mL- 120µg/mL for NAH and BUH (Fig:III,IV)) Linear Recovery values obtained by the proposed method is accurate.

The system precision was established by six replicate injections of the standard solutions containing analyte of interest. The value of relative standard deviation of Naloxone and Buprenorphine was found to be 0.72 and 0.7 within the limit, indicating the injection repeatability of the method. The method precision was established by carrying out the analysis six times using the proposed method. The relative standard deviation of Naloxone and Buprenorphine was found to be 0.6 and 0.9 within the limit, indicating the injection repeatability of the method(Table.IV).

Six samples of the same batch were prepared on different days by the analysts. Calculated %RSD for two different days in six samples for ruggedness results with the method precision within the limits. The system suitability was evaluated in each condition and compared the results with method precision results. The method is robust for change in wavelength, mobile phase composition and column oven temperature.

The specificity studies include deliberate degradation of the tablet sample by exposure to stress conditions, Specificity studies also include blank, placebo solution and sample solution (control sample), NAH and BUH standard solution were injected into the HPLC system. There was no interference from the blank and placebo at the retention time of the peaks. Peak purity data reveals that Naloxone and Buprenorphine were homogeneous and there was no interference at the retention time of both drug peaks. The method does not permit detection of any degradation products for NAH and BUH after subjecting to various degradation procedures like acid, base, thermal, peroxide and photolytic degradations, the stressed samples were analyzed by the proposed method. Degradation studies showed that the both the drugs were highly stable under acidic, oxidative, thermal and photolytic conditions without any change in RT values but under alkaline conditions RT values were shifted to lower as compared to standard without any additional peaks(Fig.VI-X).



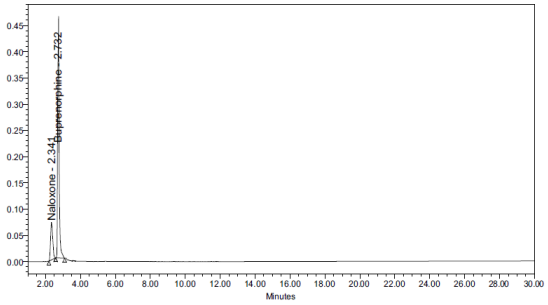
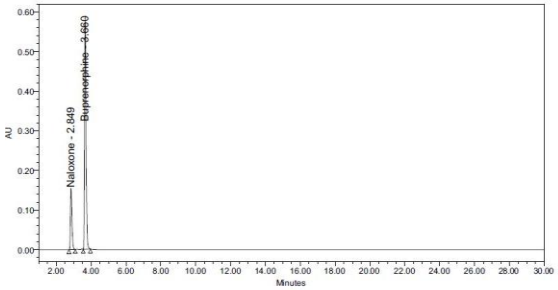


Fig VI: Chromatogram of Acid degradation **Fig VII: Chromatogram of Alkali degradation**



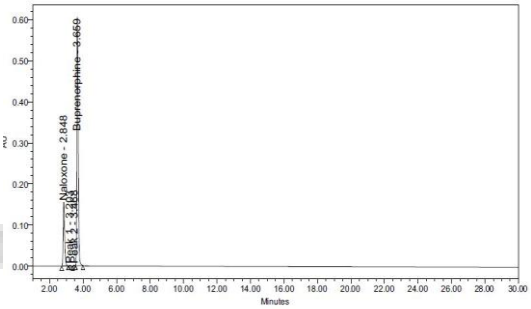


Fig VIII: Chromatogram of thermal degradation **Fig XI: Chromatogram of peroxide degradation**

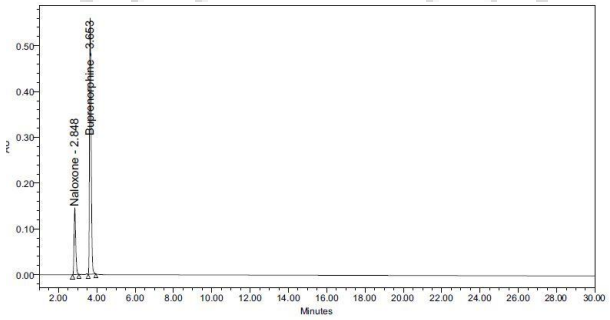


Fig X: Chromatogram of photolytic degradation

The high percentage of stability under stress conditions confirms the suitability of the method for simultaneous estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride in pure and marketed formulations. The formulation was calculated from the calibration curve. The amount of Naloxone Hydrochloride and Buprenorphine Hydrochloride in per tablet Naloxone and Buprenorphine was found to be 0.50 ± 0.009 mg and 2.03 ± 0.008 mg. Total label claim for 0.5mg Naloxone and 2mg Buprenorphine of formulation (Fig.XI).

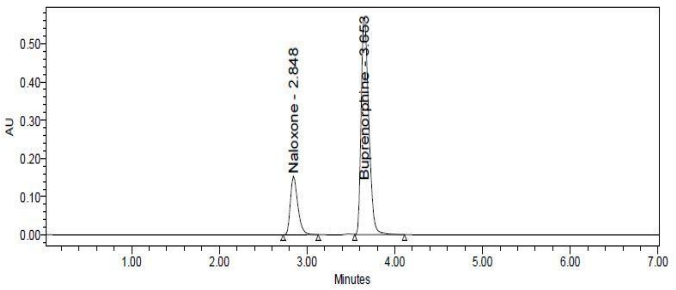


Fig XI: Chromatogram for assay of market formulation of NAH and BUH

CONCLUSION

The HPLC method developed and validated allows a simple and fast quantitative simultaneous estimation of Naloxone Hydrochloride and Buprenorphine Hydrochloride from its formulation. All the validation parameters were found to be within the limits according to the ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged, and robust and can be involved in the routine analysis of the marketed formulation. The high percentage of stability under stress conditions confirms the suitability of the method. Therefore this method can be employed in quality control to estimate the amount of Naloxone Hydrochloride and Buprenorphine Hydrochloride in pure and pharmaceutical dosage forms.

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