

Stability Indicating Analytical Method Development and Validation for the Simultaneous Determination of Degradation Impurities of Buprenorphine and Naloxone in Combination Pharmaceutical Dosage Form by RP-HPLC

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Abstract

A stability-indicating reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous quantitative determination of degradation products of Buprenorphine and Naloxone in a combination pharmaceutical dosage form. Efficient chromatographic separation was achieved using gradient elution with mixture of acetonitrile and 50 mM potassium phosphate monobasic buffer (adjusted to pH 4.5 with diluted phosphoric acid) as mobile phase A, and acetonitrile as mobile phase B. The separation was performed on a Phenomenex Luna C18 column (4.6 × 100 mm, 3 μm). UV detection was carried out at 280 nm, with flow rates of 1.1 mL/min and 1.2 mL/min. The method was validated in accordance with ICH guidelines to demonstrate specificity, linearity, limits of detection and quantitation, precision, accuracy, robustness, and solution stability. This validated method was successfully applied for the simultaneous quantitative determination of degradation products of Buprenorphine and Naloxone in the combination dosage form during both release testing and shelf-life stability studies.

Keywords

HPLC, Buprenorphine Hydrochloride, Naloxone Hydrochloride Dihydrate, Degradation Products, Combined Dosage Form, Stability Indicating Method, Forced Degradation

1. Introduction

A stability-indicating reverse phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous quantitative determination of degradation products of Buprenorphine and Naloxone in a combination pharmaceutical dosage form.

Buprenorphine hydrochloride (**Figure 1(a)**) is chemically known as (S)-2-[17-(Cyclopropylmethyl)-4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethanomorphinan-7 α -yl]-3,3-dimethylbutan-2-ol hydrochloride [1]. Its molecular formula is C₂₉H₄₁NO₄·HCl, and its molecular weight is 504.11 g/mol. Buprenorphine hydrochloride is a potent semi-synthetic opiate analgesic, with a potency 20 to 40 times greater than that of morphine [2]. It is synthesized from thebaine via a seven-step chemical process [3]. Buprenorphine hydrochloride can be administered by various routes, including parenteral and sublingual [4], and is commonly used for the management of moderate to severe acute and chronic pain [5].

Naloxone hydrochloride (**Figure 1(b)**) is chemically known as Morphinan-6-one-4,5-epoxy-3,14-dihydroxy-17-(2-propenyl) hydrochloride [6]. Its chemical formula is C₁₉H₂₁NO₄·HCl, and its molecular weight is 399.87 g/mol. Naloxone hydrochloride is a potent opioid antagonist that acts as a competitive antagonist at the μ , δ , and κ opioid receptors [7]-[9].

A combination tablet containing buprenorphine and naloxone at a fixed dose ratio of 4:1 (2 mg buprenorphine: 0.5 mg naloxone and 8 mg buprenorphine: 2 mg naloxone) has been approved by the Food and Drug Administration (FDA) for the treatment of moderate to severe pain as well as chronic pain [10] [11].

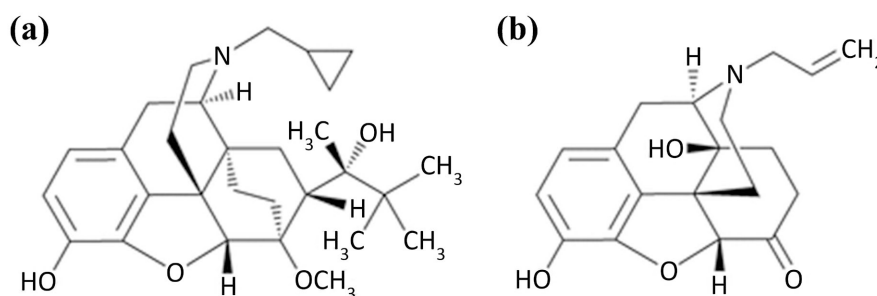


Figure 1. (a) Buprenorphine (free base), (b) Naloxone (free base).

A literature survey reveals that numerous analytical methods have been reported for the estimation of buprenorphine and naloxone, either individually or in combination with other drugs. These methods include spectroscopic techniques and chromatographic approaches such as gas chromatography with electron-capture or mass spectrometric detection, and high-performance liquid chromatography (HPLC) with fluorescence, electrochemical, or mass spectrometric detection [12]-[17]. However, no stability-indicating RP-HPLC method has been established for the simultaneous determination of known degradation products of buprenorphine and naloxone in a combination pharmaceutical dosage form.

The present work describes the development and validation of a stability-indicating RP-HPLC method capable of simultaneously quantifying known degradation products of Buprenorphine and Naloxone from a combined dosage form. In this study, two impurities of Buprenorphine and six impurities of Naloxone were used during the development of the stability indicating method. The method was validated in accordance with the International Conference on Harmonisation (ICH) Q2(R2) guideline and United States Pharmacopeia (USP) recommendations [18].

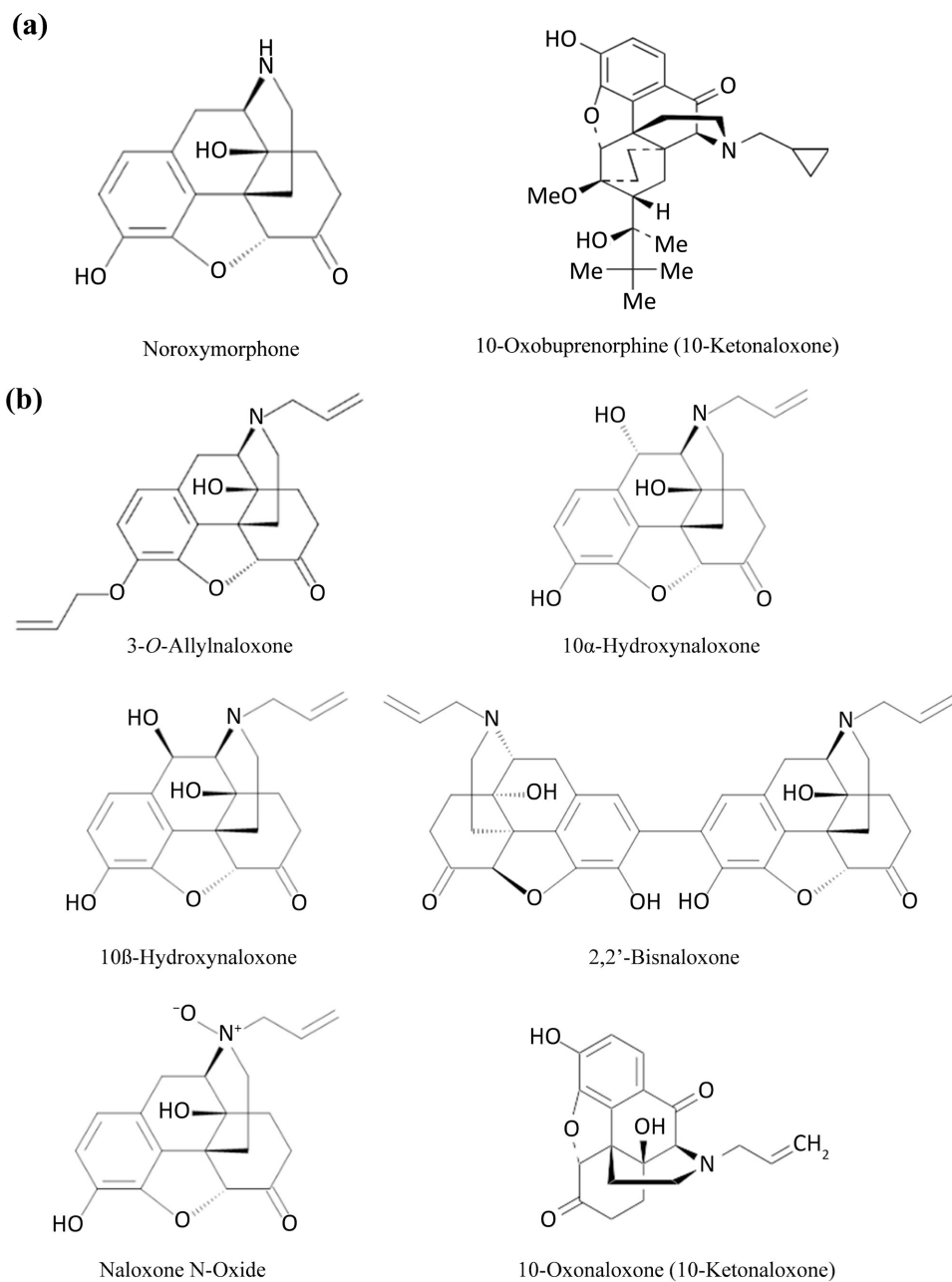


Figure 2. (a) Chemical structures of Buprenorphine impurities, (b) Chemical structures of Naloxone impurities.

Two degradation impurities of Buprenorphine are Noroxymorphone and 10-Oxobuprenorphine (**Figure 2(a)**). Six impurities of Naloxone (**Figure 2(b)**) include 3-O-Allylnaloxone, 10 α -Hydroxynaloxone, 10 β -Hydroxynaloxone, 2,2'-Bisnaloxone, Naloxone N-oxide, and 10-Oxonaloxone. Among these six impurities, three impurities are naloxone N-oxide, 3-O-Allylnaloxone, and 10-Oxonaloxone are degradation products, while the other three are related impurities. All degradation products of Buprenorphine and Naloxone were observed to increase during the stress and stability studies.

2. Method Development and Optimization for Stability-Indicating Method

A robust, stability-indicating method was developed to effectively and accurately differentiate both active pharmaceutical ingredients (APIs), Buprenorphine and Naloxone, in a combined dosage form, along with their specified and unspecified degradation products. The method was optimized to ensure adequate separation of all potential degradants formed under forced degradation conditions, as well as those originating from placebo components and process-related impurities.

Initial method development was carried out using both isocratic and gradient elution modes. Various buffer systems, including acetate and phosphate buffers at pH values of 3.5, 4.5, and 5.5, were evaluated in combination with organic modifiers such as acetonitrile and methanol. Multiple stationary phases, including C18, C8, Phenyl-Hexyl, HILIC (Hydrophilic Interaction Liquid Chromatography), and Cyano columns, were screened to resolve closely eluting peaks, particularly Naloxone N-oxide from Naloxone and Norbuprenorphine from 3-O-Allylnaloxone. However, satisfactory separation could not be achieved using C8, Phenyl-Hexyl, HILIC, and Cyano columns. The UV detection wavelength of 280 nm was selected to correspond to the absorption maxima (λ -max) of Buprenorphine, Naloxone, and their related substances, thereby ensuring optimal sensitivity, linearity, accuracy, and precision. Also by considering the minimized interference from placebo/excipient components, supporting reliable and accurate quantitation.

Ultimately, optimal separation was achieved using a Phenomenex Luna C18 column (4.6 \times 100 mm, 3- μ m particle size), employing a gradient method with potassium phosphate buffer at pH 4.5 \pm 0.1 as Mobile Phase A and acetonitrile as Mobile Phase B. A flow rate of 1.1 to 1.2 mL/min at wavelength 280 nm was found to be optimal. Under these conditions, all critical pairs including degradation products, placebo interferences, and the active substances were effectively resolved within a 35-minute runtime.

Materials and Experimental

Reference Standards:

Buprenorphine (USP Reference Standard), Naloxone (USP Reference Standard), Buprenorphine impurity standards (Purisys and LGC Standards) and Naloxone impurity standards (Purisys and LGC Standards) were used throughout the experiments.

Chemicals and Reagents:

Acetonitrile of HPLC grade was obtained from Honeywell Chemicals. Potassium dihydrogen phosphate and phosphoric acid, both of ACS grade, were procured from Sigma-Aldrich. Ultrapure water was prepared using the Milli-Q[®] Benchtop Lab Water Purification System.

Instrumentation:

The Shimadzu LC-2050c and Waters ACQUITY ARC HPLC systems, equipped with UV-Visible and photodiode array (PDA) detectors, were used for analysis. Chromatographic data were collected and processed using Empower software.

Chromatographic Conditions:

Chromatographic separation was achieved using a Phenomenex Luna C18 column (4.6 × 100 mm, 3 μm) maintained at a column oven temperature of 35 °C. The mobile phase consisted of 50 mM phosphate buffer at pH 4.5 ± 0.1 (mobile phase A) and acetonitrile (mobile phase B). Gradient elution was applied with a flow rate of 1.1 mL/min for the first 3 minutes, increased to 1.2 mL/min until 30 minutes, and then returned to the initial flow rate of 1.1 mL/min, as outlined in **Table 1**. The injection volume was 50 μL, and analytes were detected at 280 nm. The total run time was 35 minutes.

Table 1. Gradient conditions.

Time (Minutes)	Flow (mL/min)	Mobile Phase "A" (%)	Mobile Phase "B" (%)
0	1.1	95	5
3	1.2	92	8
12	1.2	75	25
18	1.2	50	50
25	1.2	38	62
30	1.2	38	62
30.1	1.1	95	5
35	1.1	95	5

Diluent Preparation:

Diluted phosphoric acid was prepared by diluting 1.0 mL of phosphoric acid to 1000 mL with water.

Preparation of Standard Solution:

The standard solution was prepared in diluent to contain Buprenorphine at a concentration of 5.2 μg/mL and Naloxone at 1.3 μg/mL.

Preparation of Sensitivity Solution:

The sensitivity solution was prepared in diluent to contain Naloxone at 1.04 μg/mL and Buprenorphine at 0.26 μg/mL.

Preparation of Sample Solution:

Thirteen tablets, each containing 8 mg of Buprenorphine and 2 mg of Nalox-

one, were transferred into a 100 mL volumetric flask. Approximately 50 mL of diluent was added, and the contents were sonicated for 15 minutes with intermittent swirling. The volume was then made up to 100 mL with diluent. The solution was centrifuged at 10,000 rpm for 5 minutes to obtain a clear supernatant, yielding a final sample concentration of 1.04 mg/mL for Buprenorphine and 0.26 mg/mL for Naloxone.

3. Method Validation Study

The developed stability-indicating method was validated according to standard analytical guidelines, evaluating the following parameters: specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision (repeatability and ruggedness), robustness, and solution stability for both standard and sample solutions.

Specificity:

Specificity is the ability to unequivocally assess the analyte in the presence of components that may be expected to be present, such as impurities, excipients, or degradation products within the sample matrix.

To evaluate the specificity of the method, the following solutions were prepared and injected: mobile phase, diluent, individual impurity solutions, sensitivity solution, standard solution, placebo solution, drug product sample solution, and spiked drug product sample solution containing all known Buprenorphine and Naloxone impurities. The acceptance criteria required that no interference should be observed from the diluent, mobile phase, or placebo at the retention times of Buprenorphine, Naloxone, or any of their impurities. Additionally, all impurity peaks must be well resolved from each other and from the Buprenorphine and Naloxone peaks, with a minimum USP resolution of not less than 1.2.

Table 2. Retention time and relative retention time of Buprenorphine and Naloxone impurities.

Peak Name	Retention Time (minutes)	Relative Retention Time
10 α /10 β -Hydroxynaloxone	1.721	0.107
Naloxone	2.127	0.133
2,2'-Bisnaloxone	2.411	0.150
Naloxone N-Oxide	3.146	0.196
Norbuprenorphine	8.859	0.552
3-O-Allyl Naloxone	9.327	0.582
10-Ketonaloxone	12.553	0.783
Buprenorphine	16.035	1.000
10-Ketobuprenorphine	26.671	1.663

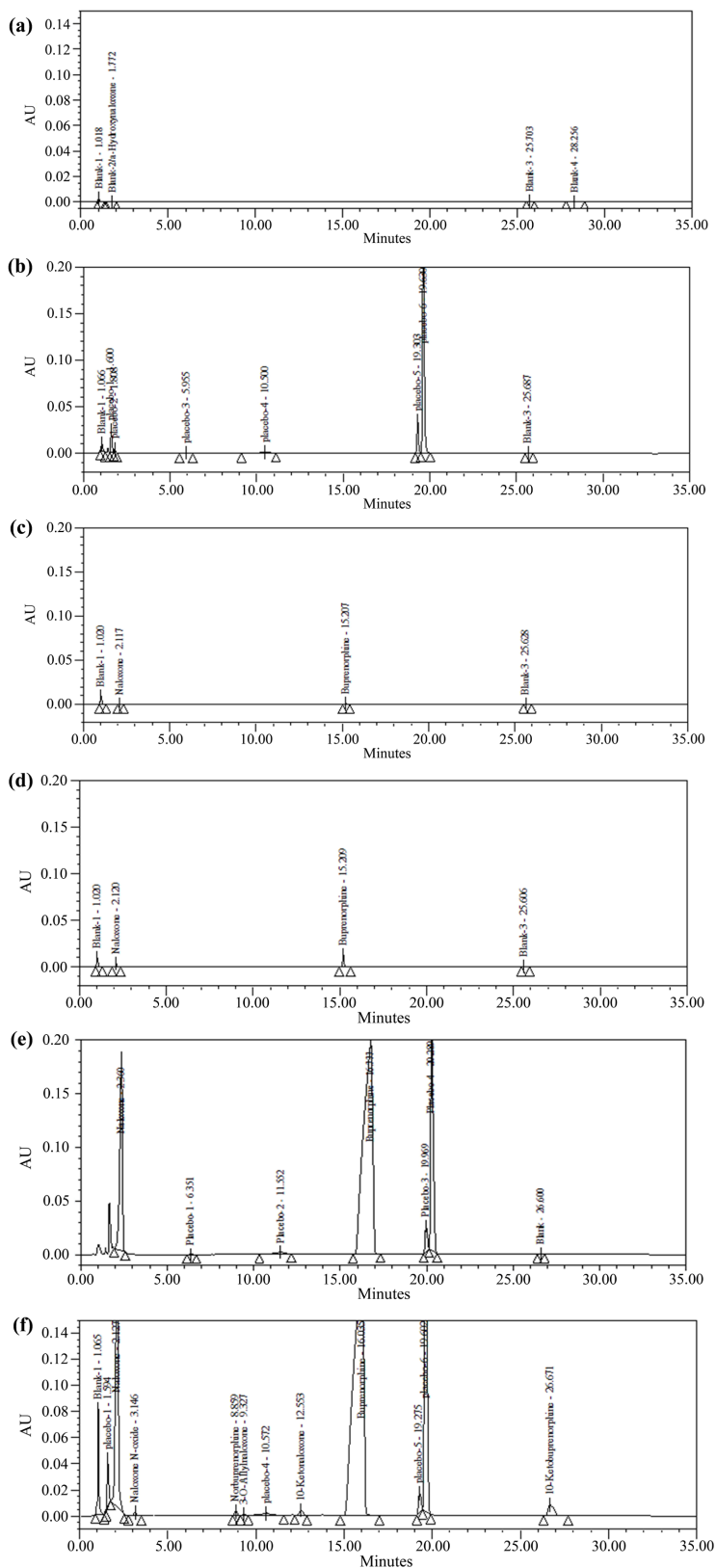


Figure 3. (a) Diluent chromatogram, (b) Placebo chromatogram, (c) Sensitivity solution chromatogram, (d) Standard solution chromatogram, (e) Drug product sample chromatogram, (f) Spiked drug product sample chromatogram.

Table 3. Stress study results for Buprenorphine in the drug product.

Stress condition	Nor-buprenorphine	10-Ketobuprenorphine	Single Largest unknown Impurity	Total Impurities
Control	<LOQ	<LOQ	<LOQ	<LOQ
Acidic	3.569	<LOQ	0.868	4.437
Alkaline	<LOQ	1.265	0.452	1.717
Oxidation	0.289	5.829	0.634	6.752
Thermal	Not detected	<LOQ	<LOQ	<LOQ
Photolytic	<LOQ	<LOQ	<LOQ	<LOQ

Table 4. Stress study results—mass balance results for Buprenorphine in the drug product.

Stress Condition	Assay (%)	Total Impurities	Mass balance (%)
Control	99.3	<LOQ	n/a
Acidic	93.7	4.437	98.8
Alkaline	95.5	1.717	97.9
Oxidation	93.4	6.752	100.9
Thermal	98.9	<LOQ	99.6
Photolytic	99.1	<LOQ	99.8

Table 5. Stress study results for Naloxone in the drug product.

Stress condition	Naloxone N-oxide	10-Ketonaloxone	3-O-Allylnaloxone	Single Largest unknown Impurity	Total Impurities
Control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Acidic	0.229	<LOQ	0.868	0.246	1.343
Alkaline	<LOQ	1.230	0.283	0.502	2.052
Oxidation	7.289	0.268	<LOQ	0.234	7.791
Thermal	Not detected	<LOQ	<LOQ	<LOQ	<LOQ
Photolytic	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 6. Stress study results—mass balance results for Naloxone in the drug product.

Stress Condition	Assay (%)	Total Impurities	Mass balance (%)
Control	98.9	<LOQ	n/a
Acidic	96.8	1.343	99.2
Alkaline	95.9	2.052	99.0
Oxidation	88.5	7.791	97.4
Thermal	97.5	<LOQ	98.6
Photolytic	98.3	<LOQ	99.4

Forced degradation:

Forced degradation studies were conducted to assess the stability-indicating nature of the analytical method by exposing the drug product and related samples to

stress conditions designed to produce approximately 5 - 20% degradation of the active pharmaceutical ingredients [19]-[24]. This degradation range helps avoid over-stressing, which could lead to irrelevant degradation products, and under-stressing, which may fail to generate degradants seen in long-term stability studies [20]. Sample solutions including placebo, placebo spiked with Buprenorphine, placebo spiked with Naloxone, and the drug product were subjected to various stress conditions: acidic hydrolysis using 10 N HCl at 80°C for 2 hours, alkaline hydrolysis using 5 N NaOH at 80°C for 2 hours, oxidation with 30% hydrogen peroxide at 80°C for 2 hours, thermal degradation at 80°C for 3 days, and photolytic degradation with exposure to 1.2 million lux hours. After the exposure period, all samples were analyzed using the validated chromatographic method, and the percentage of individual impurities and total impurities for Buprenorphine and Naloxone was calculated for each condition.

Linearity:

Linearity was evaluated by preparing a series of diluted solutions containing Buprenorphine, Naloxone, and Buprenorphine degradation products such as Nor-buprenorphine, 10-Ketobuprenorphine, Naloxone degradation products such as Naloxone N-oxide, 10-Ketonaloxone, and 3-O-Allylnaloxone at six different concentration levels. Each level was analyzed in replicate to assess the method's ability to produce results directly proportional to analyte concentration. The acceptance criteria required that the correlation coefficient (R^2) for each analyte be not less than 0.99, and the relative standard deviation (RSD) of replicate injections at each concentration level not exceed 10.0%.

Table 7. Linearity and range results for Buprenorphine and related degradation products.

Component	%Linearity Level (~)	Conc. ($\mu\text{g/mL}$)	Correlation coefficient
Buprenorphine	0.10	0.998	0.9939
	0.25	2.495	
	0.30	3.119	
	0.50	5.190	
	0.75	7.909	
Nor-buprenorphine	0.10	0.980	0.9999
	0.25	2.450	
	0.30	3.185	
	0.50	5.160	
	0.75	7.840	
10-Keto-buprenorphine	0.10	0.983	0.9998
	0.25	2.533	
	0.30	3.099	
	0.50	5.265	
	0.75	7.964	

Table 8. Linearity and range results for Naloxone and related degradation products.

Component	%Linearity Level (~)	Conc. ($\mu\text{g/mL}$)	Correlation coefficient
Naloxone	0.10	0.279	0.9983
	0.25	0.655	
	0.30	0.793	
	0.50	1.319	
	0.75	2.012	
Naloxone N-oxide	0.10	0.268	0.9993
	0.25	0.649	
	0.30	0.793	
	0.50	1.338	
	0.75	1.980	
10-Ketonaloxone	0.10	0.262	0.9997
	0.25	0.669	
	0.30	0.795	
	0.50	1.359	
	0.75	2.005	
3-O-Allylnaloxone	0.10	0.273	0.9995
	0.25	0.641	
	0.30	0.788	
	0.50	1.372	
	0.75	2.023	

Limit of Detection (LOD):

The Limit of Detection (LOD) for Buprenorphine, Naloxone, and their related degradation products was determined based on the signal-to-noise (S/N) ratio obtained during analysis. The S/N ratio for each component at the LOD should be not less than 3 and not more than 9.

Table 9. LOD results for Buprenorphine and Naloxone related degradation products.

Component	LOD Level (%)	Concentration ($\mu\text{g/mL}$)	S/N (Average)
Naloxone N-oxide	0.02	0.053	5
10-Ketonaloxone	0.02	0.056	4
3-O-Allylnaloxone	0.02	0.062	5
Nor-buprenorphine	0.02	0.210	5
10-Ketobuprenorphine	0.02	0.226	4

Limit of Quantitation (LOQ):

The Limit of Quantitation (LOQ) for Buprenorphine, Naloxone, and their related degradation products was established based on the signal-to-noise (S/N) ratio. The S/N ratio for each component at the LOQ should be not less than 10, and the relative standard deviation (RSD) should be not more than 10%, ensuring acceptable precision at the quantitation limit.

Table 10. LOQ results for Buprenorphine and Naloxone related degradation products.

Component	LOD Level (%)	Concentration ($\mu\text{g/mL}$)	S/N (Average)
Naloxone N-oxide	0.1	0.252	68
10-Ketonaloxone	0.1	0.286	128
3-O-Allylnaloxone	0.1	0.275	92
Nor-buprenorphine	0.1	1.065	125
10-Ketobuprenorphine	0.1	1.038	78

Accuracy:

The Accuracy solutions were prepared in triplicate by spiking related degradation impurities to the drug product from 0.1% to 0.75% concentration levels.

Table 11. Accuracy results for Buprenorphine related degradation products.

Accuracy Level	%Recovery	
	Nor-Buprenorphine	10-Ketobuprenorphine
0.1% - 1	105.1	97.3
0.1% - 2	105.3	97.0
0.1% - 3	106.0	96.8
0.5% - 1	103.4	100.5
0.5% - 2	103.7	100.4
0.5% - 3	103.6	100.5
0.7% - 1	104.4	102.6
0.7% - 2	104.3	102.5
0.7% - 3	104.3	102.8
Average	104.5	100.0
Range	103.4 - 106.0%	96.8 - 102.8%
SD	0.9	2.5
%RSD	0.8	2.4

Table 12. Accuracy results for Naloxone related degradation products.

Accuracy Level	%Recovery		
	Naloxone N-Oxide	10-Ketonaloxone	3-O-Allylnaloxone
0.1% - 1	99.8	108.2	97.3
0.1% - 2	101.7	103.4	97.0
0.1% - 3	102.7	103.2	96.8
0.5% - 1	104.0	99.4	100.5
0.5% - 2	105.7	99.6	100.4
0.5% - 3	104.6	99.3	100.5
0.7% - 1	103.6	101.3	102.6
0.7% - 2	103.1	101.5	102.5
0.7% - 3	103.7	101.6	102.8
Average	103.2	101.9	100.0
Range	99.8 - 105.7%	99.3 - 108.2%	96.8 - 102.8%
SD	1.7	2.8	2.5
%RSD	1.7	2.7	2.4

Table 13. Precision results for Buprenorphine and Naloxone related degradation products.

Impurity Name	%Relative Standard Deviation (n = 6 samples)		Overall %RSD (Analyst 1 & 2)	Relative Difference between Analyst 1 & 2
	Method Precision (Repeatability)	Intermediate Precision		
Naloxone N-Oxide	0.6	3.1	6.7	6.7
10-Ketonaloxone	0.2	1.0	8.1	5.6
3-O-Allylnaloxone	0.4	0.8	3.9	8.5
Nor-Buprenorphine	0.2	0.3	2.3	2.3
10-Ketobuprenorphine	0.3	0.3	1.6	2.8

Precision:

The precision of the method was evaluated through system precision, method precision (repeatability), and intermediate precision (ruggedness). System precision was assessed by preparing and analyzing the standard solution of Buprenorphine and Naloxone, with acceptance criteria that the theoretical plates for both peaks should be not less than 2000, the tailing factor should be not more than 2.0, and the relative standard deviation (RSD) of peak areas from six replicate injections should be not more than 5.0%. Method precision was evaluated by preparing six replicate drug product sample solutions spiked with all related degradation impurities. The acceptance criterion for method precision was that the RSD of individ-

ual percentage impurities from the six sample preparations should be not more than 10.0%. Intermediate precision was assessed by repeating the method precision study with a second analyst on a different day using a different instrument. The acceptance criteria for intermediate precision were that the RSD of individual percentage impurities from both analysts should be not more than 10.0%, and the relative difference between the mean impurity values obtained by the two analysts should be not more than 20.0%.

Table 14. Robustness results for sensitivity solution.

Robustness Condition	Buprenorphine	Naloxone
	S/N Ratio	
Normal	45	14
Column Temp. 30°C	55	17
Column Temp. 40°C	52	14
Flow rate 1.1 mL/min	51	15
Flow rate 1.3 mL/min	80	26
Buffer pH 4.4	43	16
Buffer pH 4.6	50	19
-5% Aqueous in mobile phase A	38	12
+5% Aqueous in mobile phase A	42	15
-5% Organic in mobile phase A	56	18
+5% Organic in mobile phase A	38	16

Table 15. Robustness results for standard solution.

Robustness Condition	Buprenorphine		Naloxone	
	%RSD	USP Tailing Factor	%RSD	USP Tailing Factor
Normal	0.2	1.1	1.0	1.0
Column Temp. 30°C	0.0	1.1	1.0	1.0
Column Temp. 40°C	0.1	1.1	0.7	0.9
Flow rate 1.1 mL/min	0.1	1.1	1.0	1.0
Flow rate 1.3 mL/min	0.2	1.1	1.1	0.9
Buffer pH 4.4	0.3	1.0	0.8	1.0
Buffer pH 4.6	0.2	1.2	0.6	0.9
-5% Aqueous in mobile phase A	0.2	1.0	1.0	0.9
+5% Aqueous in mobile phase A	0.1	1.1	0.9	1.0
-5% Organic in mobile phase A	0.2	1.1	1.0	0.9
+5% Organic in mobile phase A	0.2	1.1	1.0	0.9

Table 16. Robustness results for Buprenorphine related impurities solution.

Robustness Condition	Nor-Buprenorphine		10-Ketobuprenorphine	
	%Impurity	%Relative Difference from Normal	%Impurity	%Relative Difference from Normal
Normal	0.475	n/a	0.451	n/a
Column Temp. 30 °C	0.435	-11.5	0.420	-16.0
Column Temp. 40 °C	0.468	-3.6	0.447	-8.9
Flow rate 1.0 mL/min	0.461	-5.2	0.420	-16.0
Flow rate 1.3 mL/min	0.466	-4.1	0.468	-4.1
Buffer pH 4.4	0.469	-3.4	0.464	-5.0
Buffer pH 4.6	0.462	-5.0	0.492	1.0
-5% Aqueous in mobile phase A	0.482	-0.6	0.488	0.2
+5% Aqueous in mobile phase A	0.463	-4.8	0.473	-3.0
-5% Organic in mobile phase A	0.487	0.4	0.489	0.4
+5% Organic in mobile phase A	0.492	1.4	0.492	1.0

Table 17. Robustness results for Naloxone related impurities solution.

Robustness Condition	Naloxone N-Oxide		10-Ketonaloxone		3-O-Allylnaloxone	
	%Impurity	%Relative Difference from Normal	%Impurity	%Relative Difference from Normal	%Impurity	%Relative Difference from Normal
Normal	0.485	n/a	0.487	n/a	0.477	n/a
Column Temp. 30 °C	0.483	-0.4	0.472	3.2	0.458	-4.1
Column Temp. 40 °C	0.490	1.0	0.461	5.6	0.469	-1.7
Flow rate 1.0 mL/min	0.514	5.6	0.474	2.7	0.484	1.4
Flow rate 1.3 mL/min	0.466	-4.1	0.477	2.1	0.497	4.0
Buffer pH 4.4	0.452	-7.3	0.468	4.1	0.458	-4.1
Buffer pH 4.6	0.455	-6.6	0.492	1.0	0.472	-1.1
-5% Aqueous in mobile phase A	0.478	-1.5	0.518	6.0	0.439	-8.7
+5% Aqueous in mobile phase A	0.453	-7.1	0.483	0.8	0.508	6.1
-5% Organic in mobile phase A	0.462	-5.0	0.459	6.1	0.469	-1.7
+5% Organic in mobile phase A	0.489	0.8	0.512	4.9	0.516	7.6

Robustness:

To demonstrate the robustness of the method, analyses of sensitivity solution,

standard solution, and spiked solution were performed by deliberately changing chromatographic conditions and mobile phase composition. The robustness was evaluated by varying the column temperature by +5°C, increasing the mobile phase flow rate by +0.1 mL/min, altering mobile phase “A” composition by +5% aqueous and +5% organic content, and adjusting the buffer pH by +0.1 units. The results were considered acceptable if system suitability criteria were met, including a signal-to-noise ratio (S/N) not less than 10, tailing factor not more than 2.0, and %RSD not more than 5.0% for sensitivity and standard injections. Additionally, the relative difference in known impurity levels in the spiked solution had to be not more than 25.0%, and the resolution between the closest eluting peaks in the spiked sample solution had to be not less than 1.2 for each changed parameter.

Solution Stability:

Standard and spiked drug product sample solutions containing all related degradation products of Buprenorphine and Naloxone were prepared and analyzed initially, and then after storage at room temperature for 24 and 48 hours. The acceptance criteria for solution stability were set as follows: the assay value of the standard solution should not deviate by more than $\pm 5.0\%$ from the initial value; the percentage relative difference of known impurities should not exceed 25% compared to the initial value; and any unknown impurities generated during storage should not increase by more than 0.05% relative to the initial measurement.

Table 18. Standard solution stability results.

Time	Buprenorphine		Naloxone	
	Assay (%)	Difference (%) from Initial	Assay (%)	Difference (%) from Initial
Initial	101.1	n/a	100.2	n/a
24 hours	98.4	1.8	99.5	0.7
48 hours	96.6	3.6	98.4	1.8

Table 19. Sample solution stability results.

Impurity Name	%Relative Difference from Initial	
	24 hours	48 hours
Naloxone N-Oxide	0.02	0.01
10-Ketonaloxone	0.02	0.02
3-O-Allylnaloxone	0.03	0.02
Nor-buprenorphine	0.02	0.02
10-Ketobuprenorphine	0.01	0.03

Method Validation:

Method validation studies were performed on the finally optimized stability-indicating RP-HPLC method in accordance with the International Conference on

Harmonization (ICH) Q2(R2) guidelines and USP recommendations. The validation parameters evaluated included system suitability, specificity, linearity, accuracy, limit of quantitation (LOQ), limit of detection (LOD), precision (repeatability and ruggedness), solution stability, and robustness.

4. Results and Discussion

Specificity:

Based on the specificity study results, no interference was observed from the diluent or placebo peaks at the retention times of the degradation impurities, Buprenorphine, and Naloxone peaks. All impurity peaks were well separated from each other and from Buprenorphine and Naloxone peaks (**Figures 3(a)-(f)** and **Table 2**). The system suitability parameters met the acceptance criteria. The developed method was demonstrated to be specific and selective for quantitation of degradation products of Naloxone and Buprenorphine in the drug product.

Forced Degradation:

Forced degradation studies performed on the combination drug product confirmed the stability-indicating nature of the method. All impurities and degradation products generated under forced degradation conditions were well separated from Buprenorphine and Naloxone peaks, confirming the method's capability to detect degradation products formed during stress conditions (**Tables 3-6**).

Linearity:

Linearity was established over the range of 0.1% to 0.75% of the nominal concentration for Buprenorphine, Naloxone, and their related degradation products. The correlation coefficients between concentration and peak area were greater than 0.99 for all components (**Tables 7-8**).

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The method sensitivity was demonstrated through the LOD and LOQ values for Buprenorphine, Naloxone, and their degradation impurities. The signal-to-noise ratios for all components at LOD levels were within acceptance criteria (see **Table 9**), and the %RSD and signal-to-noise ratios at LOQ levels also met acceptance criteria (see **Table 10**).

Accuracy:

Recovery studies showed that for all components and concentration levels, recovery values ranged from 96.8% to 108.2%, consistent with theoretical values and confirming the accuracy of the method (**Tables 11-12**). The method was concluded to be accurate over the range of 0.1% to 0.70% of the nominal concentration for Buprenorphine and Naloxone degradation products.

Precision:

The %RSD values for Buprenorphine and Naloxone peaks from six replicate injections of the standard solution were well within acceptance criteria. The %RSD values of individual degradation impurities within a single analyst, as well as %RSD and relative difference between two analysts (repeatability and intermediate precision), were not greater than 10.0% (**Table 13**), demonstrating the method's precision.

Robustness:

Robustness testing showed the method to be robust against deliberate variations in chromatographic conditions such as flow rate, column temperature, mobile phase composition (aqueous and organic content), and buffer pH (Tables 14-17).

Solution Stability:

Solution stability studies demonstrated that standard and spiked sample solutions were stable at room temperature for up to 48 hours (Tables 18-19).

5. Conclusion

A rapid, accurate, and reliable stability-indicating RP-HPLC method for simultaneous quantitative determination of degradation products of Buprenorphine and Naloxone in a combined dosage form was successfully developed and validated. The validation covered specificity, linearity, LOD, LOQ, precision, accuracy, robustness, and solution stability. Three degradation products related to Naloxone (Naloxone N-Oxide, 10-Ketonaloxone, and 3-O-Allylnaloxone) and two related to Buprenorphine (Nor-buprenorphine and 10-Ketobuprenorphine) were included in the method development and validation in accordance with ICH guideline Q2(R2). To the best of our knowledge, no stability-indicating analytical method including degradation products for the combined Buprenorphine and Naloxone drug product by RP-HPLC has been reported in the literature, highlighting the originality of this study. This method is suitable for impurity testing at product release and stability studies for this pharmaceutical combination.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

- [1] United States Pharmacopeia (USP/NF) (2022) United States Pharmacopeia Monograph (USP) for Buprenorphine Hydrochloride. https://doi.org/10.31003/USPNF_M10510_06_01
- [2] Tzschentke, T.M. (2002) Behavioral Pharmacology of Buprenorphine, with a Focus on Preclinical Models of Reward and Addiction. *Psychopharmacology*, **161**, 1-16. <https://doi.org/10.1007/s00213-002-1003-8>
- [3] Heel, R.C., Brogden, R.N., Speight, T.M. and Avery, G.S. (1979) Buprenorphine. *Drugs*, **17**, 81-110. <https://doi.org/10.2165/00003495-197917020-00001>
- [4] Davis, M.P. (2005) Buprenorphine in Cancer Pain. *Supportive Care in Cancer*, **13**, 878-887. <https://doi.org/10.1007/s00520-005-0849-9>
- [5] Shulman, M., Wai, J.M. and Nunes, E.V. (2019) Buprenorphine Treatment for Opioid Use Disorder: An Overview. *CNS Drugs*, **33**, 567-580. <https://doi.org/10.1007/s40263-019-00637-z>

- [6] United States Pharmacopeia (USP/NF) (2022) United States Pharmacopeia Monograph (USP) for Buprenorphine Hydrochloride. https://doi.org/10.31003/USPNF_M10510_06_01
- [7] Hoskin, P.J. and Hanks, G.W. (1991) Opioid Agonist-Antagonist Drugs in Acute and Chronic Pain States. *Drugs*, **41**, 326-344. <https://doi.org/10.2165/00003495-199141030-00002>
- [8] Michaelis, L.L., Hickey, P.R., Clark, T.A. and Dixon, W.M. (1974) Ventricular Irritability Associated with the Use of Naloxone Hydrochloride: Two Case Reports and Laboratory Assessment of the Effect of the Drug on Cardiac Excitability. *The Annals of Thoracic Surgery*, **18**, 608-614. [https://doi.org/10.1016/s0003-4975\(10\)64408-6](https://doi.org/10.1016/s0003-4975(10)64408-6)
- [9] Lutfy, K. and Cowan, A. (2004) Buprenorphine: A Unique Drug with Complex Pharmacology. *Current Neuropharmacology*, **2**, 395-402. <https://doi.org/10.2174/157015904335947>
- [10] Collins, G.B. and McAllister, M.S. (2007) Buprenorphine Maintenance: A New Treatment for Opioid Dependence. *Cleveland Clinic Journal of Medicine*, **74**, 514-520. <https://doi.org/10.3949/ccjm.74.7.514>
- [11] Bell, J., Shanahan, M., Mutch, C., Rea, F., Ryan, A., Batey, R., *et al.* (2007) A Randomized Trial of Effectiveness and Cost-Effectiveness of Observed versus Unobserved Administration of Buprenorphine-Naloxone for Heroin Dependence. *Addiction*, **102**, 1899-1907. <https://doi.org/10.1111/j.1360-0443.2007.01979.x>
- [12] Mostafavi, A., Abedi, G., Jamshidi, A., Afzali, D. and Talebi, M. (2009) Development and Validation of a HPLC Method for the Determination of Buprenorphine Hydrochloride, Naloxone Hydrochloride and Noroxymorphone in a Tablet Formulation. *Talanta*, **77**, 1415-1419. <https://doi.org/10.1016/j.talanta.2008.09.024>
- [13] Liu, S.Y., Liu, K.S., Kuei, C.H., Tzeng, J.I., *et al.* (2005) Simultaneous Determination of Buprenorphine and Its Prodrug, Buprenorphine Propionate, by High-Performance Liquid Chromatography with Fluorescence Detection. *Journal of Chromatography B*, **818**, 233-239. <https://doi.org/10.1016/j.jchromb.2005.01.002>
- [14] Pirnay, S., Hervé, F., Bouchonnet, S., Perrin, B., Baud, F.J. and Ricordel, I. (2006) Liquid Chromatographic-Electrospray Ionization Mass Spectrometric Quantitative Analysis of Buprenorphine, Norbuprenorphine, Nordiazepam and Oxazepam in Rat Plasma. *Journal of Pharmaceutical and Biomedical Analysis*, **41**, 1135-1145. <https://doi.org/10.1016/j.jpba.2006.02.020>
- [15] Rodriguez-Rosas, M.E., Lofwall, M.R., Strain, E.C., Siluk, D. and Wainer, I.W. (2007) Simultaneous Determination of Buprenorphine, Norbuprenorphine and the Enantiomers of Methadone and Its Metabolite (EDDP) in Human Plasma by Liquid Chromatography/Mass Spectrometry. *Journal of Chromatography B*, **850**, 538-543. <https://doi.org/10.1016/j.jchromb.2006.11.025>
- [16] Damodar, K. (2011) Analytical Method Development and Validation for the Simultaneous Estimation of Buprenorphine Hydrochloride and Naloxone Hydrochloride in Pharmaceutical Dosage Forms by RPHPLC. *International Journal of Pharmaceutical and Biological Archives*, **2**, 1751-1756.
- [17] Mundhey, D.A., Sapkal, N.P. and Daud, A.S. (2016) Simultaneous Quantification of Buprenorphine HCl and Naloxonehcl by Vierordt's Method. *International Journal of Pharmacy and Pharmaceutical Science*, **8**, 101-107.
- [18] International Council for Harmonization (ICH): Validation of Analytical Procedures Q2(R2).
- [19] Reynolds, D.W., Facchine, K.L., Mullaney, J.F., Alsante, K.M., Hatajik, T.D. and Motto, M.G. (2002) Available Guidance and Best Practices for Conducting Forced Degradation Studies. *Pharmacy Technician*, **26**, 48-56.

- [20] Alsante, K.M., Ando, A., Brown, R., Ensing, J., Hatajik, T.D., Kong, W. and Tsuda, Y. (2007) The Role of Degradant Profiling in Active Pharmaceutical Ingredients and Drug Products. *Advanced Drug Delivery Reviews*, **59**, 29-37.
<https://doi.org/10.1016/j.addr.2006.10.006>
- [21] Ruan, J., Tattersall, P., Lozano, R. and Shah, P. (2009) The Role of Forced Degradation Studies in Stability Indicating HPLC Method Development. *American Pharmaceutical Review*, **9**, 46-53.
- [22] O'Neill, R., Yoo, O., Burcham, P., Nguyen, M. and Lim, L.Y. (2025) Development and Validation of a Stability-Indicating RP-HPLC Method for Edaravone Quantification. *Molecules*, **30**, Article 2866. <https://doi.org/10.3390/molecules30132866>
- [23] Zhang, L.X., Li, X.Y., Zhang, J.S., *et al.* (2025) A Validated, Stability-indicating HPLC Method for the Simultaneous Determination of Five Related Substances in Liraglutide Drug Substance. *Biomedical Chromatography*, **39**, e70118.
<https://doi.org/10.1002/bmc.70118>
- [24] Marie, A.A., Yassin, M.G. and Elshenawy, E.A. (2025) Stability Indicating RP-HPLC Method for Estimation of Finerenone and Its Related Substances in New Dosage Form. *Scientific Reports*, **15**, Article No. 20229.
<https://doi.org/10.1038/s41598-025-07166-4>