ABSTRACT

Objective: The objective of the study was to develop a stability indicating high-performance liquid chromatographic (HPLC) method for simultaneous assay of pentazocine and naloxone in bulk and tablets.

Methods: Pentazocine and naloxone were analyzed on Dionex C18 column using 0.1M K$_2$HPO$_4$ buffer (pH 4.0) and methanol (60:40, v/v) as the mobile phase. The concentration of pentazocine and naloxone was quantified by photodiode array detector set at 248 nm. The method was validated in compliance with ICH rules. Pentazocine and naloxone tablet formulation was subjected to forced degradation such as acid, neutral and alkaline hydrolysis, oxidation, photo, and thermal degradation.

Results: The method was linear, with R$^2=0.9999$ in the concentration range 100–300 μg/ml for pentazocine and R$^2=0.9995$ in the concentration range 1–3 μg/ml for naloxone. The level of detection and quantification was 0.097 μg/ml and 0.322 μg/ml for pentazocine and 0.0073 μg/ml and 0.0243 μg/ml for naloxone, respectively. The degraded products are resolved well from pentazocine and naloxone with significantly different retention time values. From validation results, it was proved that the method is selective, precise, robust, and accurate for the estimation of pentazocine and naloxone simultaneously.

Conclusion: The developed stability-indicating HPLC method can be applied for quantitative determination of pentazocine and naloxone in tablets.

Keywords: Pentazocine, Naloxone, Synthetic opioids, Degradation, Reverse-phase high-performance liquid chromatographic.

INTRODUCTION

Pentazocine, chemically known as (2RS,6RS,11RS)-6, 11-dimethyl-3-(3-methylbut-2-en-1-yl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol (Fig 1), is a synthetic opioid. It has agonist activity at K and δ opioid receptors and antagonist activity at μ opioid receptor [1-3]. Pentazocine is used as a pain reliever for moderate to severe pain [3-5].

Naloxone, chemically known as (4R,4aS,7aR,12bS)-4a,9-dihydroxy-3-prop-2-enyl-2,4,5,6,7a,13-hexahydro-1H-4,12-methano-3-benzofuro[3,2,1-e]isoquinoline-7-one (Fig 1), is a synthetic N-Acetyl oxymorphone derivative with opiate antagonist activity [6,7]. It has antagonist activity at K, μ, and δ opioid receptors. Naloxone is used in emergency cases to reverse respiratory depression due to overdoses of opioids such as morphine, heroin, and other opioids [8-11].

The fixed-dose combination of pentazocine and naloxone was approved by the FDA [12]. The combination of pentazocine and naloxone is used to reduce pain that is extreme requiring opioid therapy as it may not be tolerated or other pain medications have not worked well enough [13,14]. Pentazocine was quantified in pharmaceutical samples [15-17], human serum [18], human plasma [19-21], human urine [21,22], and whole human blood [22] using visible spectrophotometry [15,16], Thin-layer chromatography [17], high-performance liquid chromatographic (HPLC) [18-20], potentiometry [21], and gas chromatography [22]. Naloxone was quantified in microplates [23], dosage forms [24], transdermal formulations [25], human plasma [26-28], human urine [28], and human liver microsomes [28] using spectrophotometry [23], HPLC [23-27], and liquid chromatography-mass spectrometry [28].

Until now, no technique was published regarding the simultaneous estimation of pentazocine and naloxone. The present research therefore focuses on development of reverse-phase HPLC (RP-HPLC) method followed by validation as per the ICH guidelines.

MATERIALS AND METHODS

Instrumentation

HPLC analysis of pentazocine and naloxone was performed using Waters HPLC Alliance system (Waters Corporation, USA) equipped with a four-solvent quaternary pump, degasser, auto sampler, photodiode detector, and column oven. The processing of data was done using empower 2 software.

Materials

Pentazocine (98% purity) and naloxone (98% purity) were procured from Rainbow Pharma Training Lab (Hyderabad, India). The organic solvent (methanol) and chemicals (K$_2$HPO$_4$, NaOH, HCl, H$_2$O$_2$, and H$_3$PO$_4$) were of HPLC and analytical grade, respectively. Pentazocine and naloxone combination tablets (Lupin Pharmaceuticals, Inc., Baltimore) labeled to contain 50 mg of pentazocine and 0.5 mg of naloxone was obtained from a local pharmacy store (Hyderabad, India).

Chromatography conditions

The column was a C18 Dionex column (250 mm × 4.6 mm, 5 μm particle size; Thermo Fisher Scientific, US). The column temperature was kept at ambient (25°C). The mobile phase consisted of K$_2$HPO$_4$ buffer (0.1 M, pH 4.0 units, adjusted with H$_3$PO$_4$) and methanol (60:40, v/v). The flow rate and runtime were kept at 1.0 ml/min and 8 min, respectively. Detection wavelength of 248 nm was optimized. The mobile phase was used as a diluent in the preparation of solutions.

Standard solutions

Hundred mg of pentazocine and 1 mg of naloxone were correctly weighed with microbalance and dissolved in 100 ml of diluent in a...
100 ml standard flask to get a stock solution [1000 µg/ml of pentazocine and 10 µg/ml of naloxone]. This mixed stock solution was diluted aptly to get working solution with concentration 200 µg/ml of pentazocine and 2.0 µg/ml of naloxone with mobile phase.

**Calibration plot**
Calibration solutions with five different concentrations (pentazocine – 100, 150, 200, 250, and 300 µg/ml; and naloxone – 1.0, 1.5, 2.0, 2.5, and 3.0 µg/ml) were prepared from mixed stock solution by apt dilution with diluent. These solutions were analyzed using the conditions given above (section – “chromatography conditions”). The calibration plot for pentazocine and naloxone was constructed separately by plotting the peak areas against the concentrations. The method’s linearity was evaluated by determining the regression coefficient (R). Pentazocine and naloxone content in unknown samples were computed by referencing them to their respective calibration plots.

**Tablet analysis**
Pentazocine and naloxone combination tablets were grinded into powder. An appropriate weight of tablet powder equal to 200 mg of pentazocine and 2 mg of naloxone was taken, dissolved in 30 ml of mobile phase and sonicated for 20 min. The volume was then completed to 100 ml with mobile phase (2000 µg/ml of pentazocine and 20 µg/ml of naloxone). One ml of prepared solution was diluted with diluent to 10 ml (200 µg/ml of pentazocine and 2 µg/ml of naloxone) for analysis by the proposed method. Pentazocine and naloxone contents in the tablets were computed by referencing them to their respective calibration plots.

**Stress-induced degradation**
The stress conditions used to induce degradation are as follows [29]:
- Neutral hydrolysis
- Base hydrolysis
- Acid hydrolysis
- Oxidation
- Thermal
- Photolytic

The degradation was performed by adding 10 ml of reagent to 10 ml of tablet solution (2000 µg/ml pentazocine and 20 µg/ml naloxone). Hydrochloric acid (0.1 N), sodium hydroxide (0.1 N), deionized water, and hydrogen peroxide (30%) were used as reagent for acid hydrolysis, base hydrolysis, neutral hydrolysis, and oxidation, respectively. The solutions were sonicated at 25±2°C for 30 min. The samples were transmitted to a volumetric flask (100 ml) and filled with diluent to 100 ml. Following degradation, samples are filtered with a membrane filter of 0.45 µm pore size. Thermal stress was accomplished by exposing tablet powder (200 mg pentazocine and 2 mg naloxone) to 105°C for 6 h. Photolytic stress was accomplished by exposing tablet powder (200 mg pentazocine and 2 mg naloxone) to sunlight for 24 h. The thermal and photo degraded tablet sample solution was prepared as described earlier (section – “tablet analysis”) and filtered with a membrane filter of 0.45 µm pore size. The suggested HPLC method was then used to analyze each sample. The peak purity of pentazocine and naloxone in stressed samples was evaluated by the photodiode array detector.

**RESULTS AND DISCUSSION**

**Method development**
Different combinations of methanol and buffer (0.1% phosphoric acid buffer, 0.1M Na₂HPO₄ buffer, and 0.1M K₂HPO₄ buffer) with different pH, as well as different flow rates, were tested. Different C18 stationary phases (Waters, Develops, Sun尼斯, and Dionex) with dimension 250 mm×4.6 mm and 5 µm particle size were tested. Best peak width, peak symmetry, resolution, and sensitivity were obtained with Dionex C18 column, mobile phase mixture with 0.1M K₂HPO₄ buffer (pH 4.0), and methanol in the ratio of 60:40 (v/v). The optimized flow rate was 1.0 ml/min and detection wavelength was 240 nm. The optimized conditions showed a rapid and good separation of pentazocine and naloxone with retention time 3.714 min–4.761 min, respectively (Fig 2).

**Method validation**
Method validation was carried out as per the ICH regulations [30,31].

**Selectivity**
During selectivity check, diluent blank, placebo, and tablet sample solution were screened and compared with standard solution for interference at retention times of pentazocine and naloxone. Significant interference was not observed at retention times of pentazocine and naloxone in the chromatograms of diluent blank, placebo, and tablet sample solution (Fig 3). Thus, selectivity was demonstrated.

**System suitability**
The parameters regarding system suitability were tested by analysis (n=5) of pentazocine and naloxone standard solution at a concentration of 200 µg/ml and 2 µg/ml, respectively. The results were found within the acceptance criteria in line with ICH directives (Table 1).

**Linearity**
Linearity of pentazocine and naloxone was achieved over the range of 100–300 µg/ml and 1–3 µg/ml, respectively. Linear regression equation and regression coefficient were calculated and given below:
- Pentazocine: Peak area = 76697 x – 12761, regression coefficient, R²=0.9999
- Naloxone: Peak area = 14621 x – 246.8, regression coefficient, R²=0.9995.

The good linearity of the method for pentazocine and naloxone was demonstrated through regression coefficient values (>0.999).

**Limit of detection (LOD) and limit of quantitation (LOQ)**
LOD and LOQ were assessed based on the standard deviation (SD) of intercept and slope (m) of the calibration plot. The below equations were employed to compute the LOD and LOQ values.

$$\text{LOD} = \text{SD} / m \times 3.3$$

$$\text{LOQ} = \text{SD} / m \times 10$$

The LOD and LOQ were 0.097 µg/ml and 0.322 µg/ml for pentazocine and 0.0073 µg/ml and 0.0243 µg/ml for naloxone, respectively. Values lesser than 1 µg/ml confirm that the developed method was sensitive adequately.

**Precision**
The precision was appraised by analyzing (n=6) pentazocine and naloxone standard solution at a concentration of 200 µg/ml and 2 µg/ml, respectively, on the same day [31,32]. The mean peak area along with the relative SD was determined (Table 2). The results were found to be within the acceptance criteria (percent relative standard deviation [RSD] value <2.0%).

**Accuracy**
The accuracy was appraised by recovery study through standard addition method [32,33]. The percent recovery studies for pentazocine and naloxone were done by spiking three different amounts of pentazocine and naloxone standard (50, 100, and 150%) to the pre-analyzed tablet sample. The percent recovery of pentazocine and naloxone was determined in three replicates for each level (Tables 3 and 4). The results of percent recovery were found within the acceptance criteria (80–120%).
Robustness

Five variation parameters were studied to demonstrate robustness:

- Change in ratio of methanol by ±5.0%
- Change in pH of buffer by ±0.1 unit
- Change in flow rate by ±0.1 ml/min
- Change in detection wavelength by ±2 nm
- Change in column temperature by ±2.0°C.

The peak area, plate count, resolution, and tailing factor values of pentazocine and naloxone obtained from variation parameters were...
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The times of elution of pentazocine, naloxone, and degradants are different. These findings proved that the method is stability indicating and specific.

Table 2: Precision data for pentazocine and naloxone

| Inj. No.* | Peak area Pentazocine | Naloxone |
|-----------|----------------------|----------|
| 1         | 7641945              | 1464317  |
| 2         | 7655208              | 1451732  |
| 3         | 7644772              | 1462000  |
| 4         | 7646482              | 1463570  |
| 5         | 7641857              | 1451692  |
| 6         | 7653061              | 1450658  |
| Mean**    | 7647221              | 1457328  |
| SD***     | 5675.3883            | 6590.9768|
| RSD****   | 0.074                | 0.452    |

*Injection number, **mean of five determinations, ***standard deviation, ****relative standard deviation

Table 3: Recovery data for pentazocine

| Level spiked (%) | Spiked Amount (µg/ml) | Determined Recovery (%) | Mean* (%) |
|------------------|-----------------------|-------------------------|-----------|
| 50               | 99.000                | 99.31                   | 100.31    | 100.28    |
| 100              | 99.000                | 99.20                   | 100.20    | 100.42    |
| 150              | 99.000                | 99.32                   | 100.32    | 100.44    |

*Mean of three determinations

Degradation study

Degradation investigation outcomes are shown in Table 6. Degradation of pentazocine and naloxone was seen in the stress conditions applied. Highest degradation of pentazocine and naloxone was observed in dry heat and acid conditions applied. Lowest degradation of pentazocine and naloxone was observed with neutral and peroxide conditions applied. The chromatograms of pentazocine and naloxone after degradation are presented in Fig. 4. The times of elution of pentazocine, naloxone, and degradants are different. These findings proved that the method is stability indicating and specific.

Table analysis

The method was applied to evaluate the content of pentazocine and naloxone in tablets. The recovered values were 100.237% for pentazocine and 101.013% for naloxone, indicating the method’s reliability (Table 7). The RSD values were 0.150% for pentazocine and 0.178% for naloxone (Table 7), indicating the method’s reproducibility.
A simple and rapid RP-HPLC method was developed to quantify pentazocine and naloxone in bulk and tablets. The results of validation are satisfactory and adequate to quantify pentazocine and naloxone simultaneously. The results of forced degradation studies established the specificity and stability indicating nature of the method. The method offers adequate selectivity and accuracy for routine evaluation and quality control in laboratories for pentazocine and naloxone.

### CONCLUSION

The authors declared that they have no conflicts of interest.

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**Table 4: Recovery data for naloxone**

| Level spiked (%) | Amount (µg/ml) | Recovery (%) | Mean* (%) |
|------------------|----------------|--------------|-----------|
|                  | Spiked         | Determined   |           |
| 50               | 0.980          | 0.99         | 101.15    | 101.15    |
|                  | 0.980          | 0.99         | 101.16    |           |
| 100              | 1.960          | 1.98         | 101.14    |           |
|                  | 1.960          | 1.99         | 101.14    |           |
|                  | 1.950          | 1.98         | 101.08    |           |
| 150              | 2.940          | 2.96         | 100.75    | 100.81    |
|                  | 2.940          | 2.97         | 100.90    |           |
|                  | 2.940          | 2.96         | 100.78    |           |

*Mean of three determinations

**Table 5: Robustness data for pentazocine and naloxone**

| Parameter          | Pen | Nal |
|-------------------|-----|-----|
| Retention time    |     |     |
| Plate count       |     |     |
| Tailing factor    |     |     |
| Resolution        |     |     |

**Table 6: Degradation data for pentazocine and naloxone**

| Deg. with | Pen area | Nal area | Pen % assy | Nal % assy | Pen % deg | Nal % deg |
|-----------|----------|----------|------------|------------|-----------|-----------|
| Acid      | 7243689  | 1334725  | 94.52      | 90.57      | 5.48      | 9.43      |
| Base      | 7340455  | 1361014  | 95.79      | 92.35      | 4.21      | 7.65      |
| Peroxide  | 7421869  | 1444737  | 96.85      | 98.04      | 3.15      | 1.96      |
| Heat      | 7198634  | 1355646  | 93.93      | 91.99      | 6.07      | 8.01      |
| Sunlight  | 7505340  | 1393436  | 97.94      | 94.55      | 2.06      | 5.45      |
| Water     | 7530071  | 1438596  | 98.26      | 97.62      | 1.74      | 2.38      |

Pen: Pentazocine, Nal: Naloxone, Flow 1: 0.9 ml/min, Flow 2: 1.1 ml/min, Temp 1: 23°C, Temp 2: 27°C, Ratio 1: Methanol ratio 45% by volume, Ratio 2: Methanol ratio 35% by volume, PH 1: 3.9 units, PH 2: 4.1 units, Nm 1: Wavelength 246 nm, Nm 2: Wavelength 250 nm

**Table 7: Quantification of pentazocine and naloxone in tablets**

| Statistical parameter | Label claim (mg) | Determined (mg) | Recovered (%) |
|-----------------------|------------------|-----------------|--------------|
| Pentazocine           |                  |                 |              |
| Mean*                 | 50               | 50.137          | 100.273      |
| SD**                  | -                | 0.0750          | 0.1501       |
| RSD***                | -                | 0.150           | 0.150        |
| Naloxone              |                  |                 |              |
| Mean*                 | 0.5              | 0.505           | 101.013      |
| SD**                  | -                | 0.0008          | 0.1795       |
| RSD***                | -                | 0.178           | 0.178        |

*Mean of three determinations, **standard deviation, ***relative standard deviation

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**AUTHORS’ CONTRIBUTION**

This work was done by RKK under the supervision of RS.

**CONFLICTS OF INTEREST**

The authors declared that they have no conflicts of interest.
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