Determination of nalbuphine hydrochloride in pharmaceutical formulations using diperiodateargentate(III)-rhodamine-B chemiluminescence system by flow injection analysis

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SUMMARY

A novel method for the analysis of nalbuphine hydrochloride (NAL) is reported based on its enhancement effect on diperiodateargentate(III)-rhodamine-B (Ag(III) complex-Rh-B) chemiluminescence (CL) system in aqueous sulfuric acid medium using flow injection analysis (FIA). The optimal conditions of the CL reaction were: sulfuric acid $10^{-2}$ M, Ag(III) complex $2.0 \times 10^{-4}$ M, Rh-B $2.0 \times 10^{-5}$ M, Brij-35 0.01%, sample loop volume 300 µL and flow rate of 3.0 mL/min/stream. The limit of detection (LOD) and limit of quantification (LOQ) were 0.001 and 0.003 mg/L ($S/N = 3$ and 10), linear calibration range of $5 \times 10^{-3} – 5.0$ mg/L ($R^2 = 0.9999$) and injection throughput of 150/h. The relative standard deviation (RSD) was from 0.8 – 3.2% over the range studied. The suggested technique was applied for the determination of NAL in pharmaceutical injections, compared with a reported spectrophotometric method and results obtained are found satisfactory. Based on the spectrophotometric studies, the most probable mechanism of the CL reaction has been briefly described and written schematically.

Keywords: Nalbuphine hydrochloride, Ag(III) complex, rhodamine-B, chemiluminescence, flow injection analysis, pharmaceutical formulations
Introduction
Nalbuphine (NAL), (−)-17-(cyclobutylmethyl)-4,5α-epoxymorphan-3,6α,14-triol hydrochloride (Fig. 1), is a semi-synthetic opioid drug and a derivative of oxymorphone. This analgesic drug has analgesic potency of about two-third in comparison to morphine. The main application of NAL is to relieve moderate to severe pain. The main applications of NAL include the treatment of medical disorders (acute and chronic) such as vascular headaches or migraine, biliary or renal colic, cancer and pain due to surgery. Further, it is used during obstetric analgesia. The oral administered NAL is not as effective for pain as intravenous and intravascular administered is, which is mainly because of first-pass metabolism in the gastrointestinal mucosa and/or the liver. Hence, its bioavailability of oral administered is lower compared with intravascular and intravenous.

A number of instrumental and analytical techniques are available for the measurement of NAL concentration in biological fluids and pharmaceutical formulations including spectrophotometry, spectrofluorimetry, potentiometry, voltammetry, capillary zone electrophoresis, gas chromatography (GC) and high performance liquid chromatography (HPLC). The HPLC and GC techniques possess high selectivity and sensitivity but are usually time-consuming, require pure and sometimes toxic solvents, expensive instrumentation and are not commonly used and found in laboratories. Electro-analytical methods usually have short linear dynamic range and lack of enough sensitivity.

Chemiluminescence (CL) is the measurement of light production emitted by an exothermic chemical reaction (>45 kcal/mol). CL is a powerful and attractive detection technique with fast response time, high sensitivity, simple instrumentation, very low detection limit (< µg/L) and wide linear range. Both flow injection (FI) technique and CL detection together provide a simple, cheap and reproducible means of screening a wide variety of analyts, which have been widely applied in various fields of analytical chemistry. Transition metals of higher oxidation states such as silver, copper, and gold etc. can exist in alkaline medium with polydentate ligands. Diperiodatoargentate(III) (Ag(III) complex) is a good oxidizing agent with redox potential of 1.74 V and has a wide range of applications in the study of chemical kinetics and mechanism of oxidation of both organic and inorganic chemical species. Owing to its strong oxidizability and catalytic properties, Ag(III) complex has attracted much attention in analytical chemistry. The reaction of Ag(III) complex with luminol based CL methods have been reported for the determination of hydrocortisone.

This work aims to establish a novel FI-CL method for selective and sensitive detection of NAL. Literature is devoid of any FI-CL method for the quantification of NAL in a sample. Therefore, it is of a prime importance to develop a sensitive, economical and fast method for NAL...
in pharmaceuticals. Under acidic conditions, weak CL is produced from Ag(I) complex as an oxidant with NAL while strong CL can be generated in the presence of Rh-B compound and Brij-35 surfactant. The interference study reveals that the proposed method provides a good accuracy and precision to determine NAL in pharmaceutical formulations. The mechanism that how light emission during the proposed CL reaction occurs has been schematically described.

EXPERIMENTAL

Reagents and solutions

All the reagents were of analytical reagent grade unless specified otherwise; for cleaning and preparation of solutions, deionized water (Elga, Pürelab Option, High Wycombe, Bucks, UK) was used. Nalbuphine stock solution of 1000 mg/L was prepared by dissolving 10 mg from its bulk in 10 mL deionized water in brown bottle and then stored at 4°C. Serial stock’s aliquots were diluted in Brij-35 solution (0.01% w/v) and used as a sample carrier stream. Nexus Pharma (Pvt.) Ltd. (Karachi, Pakistan) provided pure NAL as a gift and, therefore, is acknowledged.

The required quantity of rhodamine-B (Rh-B, Alfa-Acsar, Johnson Mathey group) compound was dissolved in 100 mL of 10 mM H₂SO₄ to arrange its 1.0×10⁻⁴ M stock solution in a brown bottle and stored at room temperature. More dilute solutions of Rh-B were prepared in 10 mM H₂SO₄ from this stock solution and used when needed.

To prepare 50 mL of 1.0 % (w/v) stock solutions of surfactants, 0.5 g each of Tween-20 and -80 (polyoxyethylene sorbitan monolaurate) and SDS (sodium dodecyl sulfate) obtained from Bio-Rad Laboratories, Hercules, CA, USA, CTAB (cetyltrimethylammonium bromide) and Brij-35 (polyoxyethylene lauryl ether) purchased from MP Biomedicals, Solon, OH, USA and Triton X-100 (polyethylene glycol tert-octylphenyl ether) obtained from Research Organics, Cleveland, OH, USA, was dissolved in deionized water and sonicated for complete homogenization. Appropriate dilutions of the stocks were performed to arrange working standard solutions. The stock solutions of the surfactants were protected from light.

A stock solution of sulfuric acid (2.0 M) was prepared by diluting its required volume in deionized water from commercially available 18 M stock solution. In order to make working standard solutions of the acid, deionized water was used for the dilution. The stock solutions (1 mM) of potassium permanganate (KMnO₄), rhodamine 6-G (Sigma Chemical Co, St. Louis, MO, USA) sodium sulphite, pyrogallol (Robert Johnson, prepared in deionized water), quinine sulphate (BDH Chemicals Ltd., Poole, UK) and cerium sulphate (Merck Darmstadt, Germany) (in H₂SO₄ 0.01 M) were prepared by dissolving the required quantity of each compound from their salts in...
deionized water and sulfuric acids respectively. Whenever required, appropriate dilutions of these stock solutions were performed to arrange the working standards with deionized water and H$_2$SO$_4$.

Stock solutions (1000 mg/L) of Mn(II), Cr(III), Ca(II), Co(II), Fe(II), Fe(III), Cd(II), and Zn(II) were prepared by dissolving the required amount of metal nitrate salts in deionized water and/or from atomic absorption standards (Merck, Darmstadt, Germany), nitrate, chloride, carbonate, bicarbonate, sulphate (BDH Chemicals Ltd., Poole, UK) and stock solutions (100 mg/L) of lactose, magnesium stearate, sodium acetate, polyethylene glycol, glycerine, sorbitan monopalmitate (not soluble in H$_2$O), sodium benzoate and glucose (Merck, Darmstadt, Germany) were prepared in deionized water. Working standards were prepared by serial dilutions (range studied 0.025, 0.25, 2.5 and 25 mg/L) of the stock solutions in Brij-35 (0.01 % w/v) as required for the interference study.

The diperiodatoargentate(III) ([Ag(HIO$_6$)$_2$]$^{5-}$) was synthesized in the laboratory by oxidizing argentum(I) in an alkaline medium according to the suggested procedure. In Brief, 3.24 g potassium periodate, 1.36 g silver nitrate, 3.0 g sodium persulphate, and 8.0 g potassium hydroxide were taken in a round bottom flask (250 mL volume) and 100 mL of deionized water was added. The resultant mixture was heated to boiling for about 30 minutes. The mixture was filtered through Gooch crucible. The filtrate was cooled down by an ice bath in order to remove K$_2$SO$_4$. The cold solution was again filtered. A greenish red filtrate was left to attain room temperature. To obtain the crystals of the complex, 40 mL of NaNO$_3$ solution (50% in excess) was added to the filtrate to obtain crystals of the complex. The crystals were then washed several times with deionized water until the dissolution of the complex started in deionized water. Before use, fresh working standard solutions of Ag(III) complex were prepared by dissolving the required quantity in dilute KOH solution. On characterizing the complex by UV-Visible spectrum, two peaks were obtained at 362±1 and 252±0.4 nm. In addition, the concentrations of Ag(III) complex working standard solutions were determined spectrophotometrically (Model UV-1700, Shimadzu, Japan) at 362 nm by use of the molar absorptivity $\varepsilon = 1.26 \times 10^4$ M$^{-1}$cm$^{-1}$. Similarly, diperiodatocuprate(III) and diperiodatonickelette(IV) (Cu(III) and Ni(IV) complexes) were synthesized according to the suggested procedures and their working standard solutions were freshly prepared by dissolving the required quantities of Cu(III) and Ni(IV) complexes in KOH (1.0×10$^{-3}$ M) before use. The concentrations of Cu(III) and Ni(IV) complexes working standard solutions were determined spectrophotometrically by measuring the absorbance at 415 and 410 nm (molar absorptivities $\varepsilon = 6.23\times10^3$ M$^{-1}$cm$^{-1}$ and $\varepsilon = 1.06\times10^5$ M$^{-1}$cm$^{-1}$) respectively. All solutions were refrigerated at 4 °C.

Analysis of pharmaceutical preparation
NAL containing ampoules (10 mg/mL) brand name; Kinz (Sami’s Specs Pharmaceuticals, (Pvt) Ltd., Karachi), Analin (Medicaids, Pakistan (Pvt) Ltd.), Nabin (Global Pharmaceuticals (Pvt) Ltd., Islamabad) and Exnal (Indus Pharma (Pvt) Ltd., Karachi) were collected from local market. One ampoule from each brand containing 10 mg/mL was transferred into brown bottles and made the volume to 10 mL with deionized water to obtain a solution of 1000 μg/mL of NAL. A series of working standard solutions covering the range of 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 μg/mL (5.0, 25, 50, 75, 100, and 125 μL/50 mL) were prepared into 50 mL volumetric flasks, then injected and analysed. The content of each ampoule was determined from the calibration curve or using the corresponding regression equation.

**Apparatus and procedure**

A three channel flow system used in the proposed study is shown in Fig. 2. All the reagents were propelled at a flow rate of 3.0 mL/min by a peristaltic pump (Ismatec, Switzerland). The NAL standards and samples were injected through an injection valve of six-way rotary setup (Rheodyne 5020, Anachem, Luton, UK) in Brij-35 (0.01%) stream which was merged with Rh-B (2×10⁻⁵ M in stream of H₂SO₄ 0.01 M). The merged stream was then mixed with Ag(III) complex (2×10⁻⁴ M in KOH 1 mM) stream and the CL emitted was collected in spiral flow cell (i.d. = 1.50 mm; diameter = 18.0 mm) mounted in front of an end window PMT (9798B, Electron Tubes Ltd., Ruislip, UK), connected to a power supply (Burle, HV power supply, type PF1053, USA), operated at 1000 V. All the FI-components were connected by PTFE tubing (0.8 mm i.d., Fisher, Loughborough, UK). The output of the PMT was recorded on a chart recorder (BD40, Kipp & Zonen, The Netherlands).

**Spectrophotometric method**

A spectrophotometric method⁷ was used in order to compare it with the proposed CL method for NAL. The NAL working standard solutions in the range 2.0, 5.0, 10.0, 15.0, and 20 mg/L (portions taken 20, 50, 100, 150 and 200 μL) were prepared from the NAL stock solution (10 mg in 10 mL deionized water) and were transferred to 10 mL volumetric flasks. Furthermore, 1.0 mL of NaOH (1.0 M) followed by 2.0 mL of KMnO₄ (5.0×10⁻³ M in deionized water) were added to each flask respectively stirred well and then added deionized water to make the total volume up to 10 mL. The flasks of the solutions were kept in a water bath which was thermostatically controlled and adjusted to 40±2 °C for about 20 min. Absorbance of the prepared mixture of solutions was monitored at 605 nm against a reagent blank. For this purpose, a double beam spectrophotometer (UV-1700, Shimadzu, Japan) equipped with 10 mm quartz cuvettes was used. Likewise, pharmaceutical formulations containing NAL were analysed. The amount of NAL calculated from the standard curve and the results were compared with the proposed FI-CL method.
RESULTS AND DISCUSSION

Optimum experimental conditions

To obtain a low limit of detection (LOD), high injection throughput and wide linear range, a series of optimization experiments were performed. These include the optimization of sulfuric acid, Ag(III) complex, Rh-B, and Brij-35 concentrations, flow rate, sample loop volume and PMT voltage. These analytical conditions were examined with 0.5 mg/L solution NAL and all the measurements were made in triplicate.

On the proposed CL reaction, sulfuric acid has remarkable effect on the CL signals. Therefore, the influence of several acids including sulfuric acid, hydrochloric acid, phosphoric acid and nitric acid (0.015 M) were investigated for the intensity of CL. Resultantly, sulfuric acid produced highest and stable CL signals. Most of the articles have reported the use of sulfuric acid in conjunction with Ag(III) complex. The mixture of silver ion and sulfate ion in the waste shown in the proposed manifold does not turn milky because they do not form precipitates at this concentration level, but the mixture of silver ion and chloride ion turns milky due to the formation of AgCl precipitate. These precipitates stop the emitted light to reach to the cathode of PMT. To optimize the concentration of sulfuric acid, it was investigated in the range of 0 – 75 mM. The CL enhanced remarkably with the increase in the concentration of sulfuric acid up to 10 mM and then decreased (Fig. 3A). Thus, sulfuric acid concentration of 10 mM was selected as an optimized concentration for subsequent experiments.

Ag(III) complex, Ag(HIO₆)₂⁻ (reduction potential of 1.74 V) is a powerful oxidant in alkaline medium with the nature of the oxidation of two electrons. Its molar strength affects the sensitivity of the method as well as influences the linear range of the procedure. Therefore, the effect of Ag(III) complex concentration on the CL intensity was investigated in the range of 0.5 – 3.0×10⁻⁴ M. Its 2.0×10⁻⁴ M concentration produces the maximal and most reproducible CL signals (Fig. 3B). The CL intensity decreased on further increase in the Ag(III) complex concentration, thus 2.0×10⁻⁴ M was selected as an optimized strength and used in the subsequent experiments. Ag(III) complex is not stable in neutral and acidic medium but its dilute solution shows high stability in basic medium. Therefore, the effect of concentration of KOH as a solvent for Ag(III) complex solution on the CL intensity was examined. The most reproducible CL signals were observed at 1.0×10⁻³ M of KOH and was selected and utilized for Ag(III) complex solution during subsequent experiments. During the optimization of sulfuric acid and KOH concentrations, it was observed that pH is an important parameter for CL emission of the proposed reaction. Emission of light when NAL reacts with Ag(III) complex in the presence of Rh-B was detected when reaction mixture was acidic. In the absence of sulfuric acid i.e. when the reaction mixture pH was basic, no light emission for the proposed reaction was detected by PMT.
The preliminary experiment showed that NAL could react with Ag(III) complex to produce a weak CL intensity and this CL reaction could be sensitized by a sensitizer. Therefore, the influence of several sensitizers including rhodamine G-6, rhodamine-B, quinine and fluorescein (2.0×10⁻⁵ M in sulfuric acid 0.01 M) was investigated on the CL intensity. Among these sensitizers, the maximal and stable CL signals were obtained with Rh-B (Fig. 3C). The influence of concentration of Rh-B on the CL emission was tested from 0 – 4.0×10⁻⁵ M. In this investigation, 2.0×10⁻⁵ M produced optimal CL signal. The intensity of CL signal remained almost same with further increase in the concentration as shown in Fig. 3D. Therefore, Rh-B concentration of 2.0×10⁻⁵ M (in sulfuric acid 0.01 M) was selected for further experiments.

Various surfactants are used as cleaning agents to wash/clean the inner wall of flow through cell. In addition, studies have shown that the surfactants may influence the CL emission. Due to production of micellar medium, the analyte is more soluble in a surfactant than in deionized water. Surfactants usually increase the efficiency and sensitivity of CL.⁴³,⁴⁴ In the present study, a gradual decrease was observed in CL intensity when Rh-B was used. This is probably due to adsorption of Rh-B on the inner wall of spiral flow through cell made up of glass. Thus, the influence of several surfactants on the CL intensity as well as for cleaning purpose was tested. To compare CL intensity for NAL, deionized water and various surfactants including Brij-35, Tween-20 and -80, Triton X-100, SDS, and CTAB (each 0.01% w/v) were used as sample carrier. Comparatively, the CL intensity for NAL was higher in Brij-35 than deionized water and other surfactants. (Fig.4A). Hence, the effect of concentration of Brij-35 on the CL intensity was also tested in the range of 0–0.25% (w/v). Brij-35 concentration of 0.01% (w/v) produced the maximal and reproducible CL signals and was selected for the present study (Fig.4B). As it is known that surfactant is a cleaning agent, therefore, it can prevent the Rh-B from adsorption on the inner walls of spiral flow cell. To prove it, 20 injections of 0.5 mg/L solution of NAL prepared in water when carrier was only water were injected into the proposed FI-CL manifold and the respective CL intensities were measured. As a result, gradual decrease in the CL intensities was observed, which indicates that Rh-B could be adsorbed on the inner walls of the flow through cell which self-absorbs the emitted light and prevent it from striking on the cathode of PMT. In contrast, when 20 injections of 0.5 mg/L solution of NAL prepared in 0.01% (w/v) Brij-35 aqueous medium were injected in the manifold when carrier was 0.01% (w/v) Brij-35 aqueous solution, no gradual decrease in the CL intensities was observed. From the results of these experiments, it can be concluded that the use of Brij-35 aqueous solution as a carrier and a medium for preparation of analyte working standard solutions can improve the reproducibility of the proposed FI-CL method for NAL analyses.

The sample injection loop volume, flow rates and PMT voltage may have direct influence on the determination of NAL CL emission and therefore were optimized to obtain high sensitivity,
reasonable sample throughput and for economy of reagents consumption. Sample injection volume was tested in the range of 60 – 420 µL and the maximal CL emission was recorded when injecting sample volume of 300 µL. The influence of flow rate on the CL intensity was tested in the range of 0.5 – 3.5 mL/min. The CL intensity increased with the increase in flow rate up to 3.0 ml/min. Thus it was selected as an optimum. Similarly, an increase in the intensity of CL was also observed by increasing the PMT voltage in the range of 800 – 1250 V. However, to avoid PMT damage voltage of 1000 V was selected and used.

Analytical performance of the method

Under the selected optimal parameters (chemical and physical), a linear calibration curve of the CL response versus NAL concentration was obtained. It was linear in the range of 0.005-5.0 mg/L ($R^2 = 0.9999$); LOD of 1.0×10^{-3} mg/L ($S/N = 3$) and LOQ of 3.0×10^{-3} mg/L ($S/N = 10$) which was calculated as the amount of NAL required to yield CL signals three and ten times the signal to noise ratio respectively and regression equation of $b = 85.325a + 3.7996$. Where $b$ is the CL intensity (mV) and $a$ is NAL concentration (mg/L). The relative standard deviation (RSD) was 0.8 – 3.2% in the range studied and sample throughput of 150/h. Chart recorder traces of CL intensity response and the calibration curve in the inset for a series of NAL standard solutions (range 0.005 – 5.0 mg/L) are given in Fig. 5.

Table 1 compares the analytical characteristics of the present method with previously reported methods for NAL in different matrices. The LOD of the proposed method is lower than that of other reported methods. However, a limited number of analytical methods e.g., voltammetric,^{13} UPLC-MS/MS,^{22} and LC-MS/MS^{23} methods are sensitive, accurate with suitable efficiency but they have some weaknesses such as, time-consuming, expensive equipment and low sample throughput. On the other hand, the proposed CL method has satisfactory linearity, high sample throughput and low detection limit comparatively.

Interference study

The effect of some common excipients (inorganic ions and several organic compounds), expected to be present in drugs, were investigated on the blank (without NAL) and on the determination of NAL (0.1 mg/L) in the concentrations range of 0.025, 0.25, 2.5 and 25 mg/L. The tolerance limit of the foreign chemical species was taken as the concentration that caused the relative error of less than ±5% in the CL intensity. The organic compounds e.g., lactose, methyl cellulose, crystalline cellulose, magnesium stearate, acetate, poly ethylene glycerol, sorbitan monopalmitate, glycerol, sodium benzoate and inorganic compounds e.g., carbonate, calcium(II), magnesium(II), chloride, nitrate, iron(II) and (III), cobalt(III) cadmium(II), zinc(II) and titanium
dioxide did not show any interference activity on the blank signals and on the NAL determination except manganese(II) and chromium(III) which had slight suppressive effect on blank and on the determination of NAL (0.1 mg/L). However, these cations are not present usually in pharmaceutical formulations. If present, they can be removed easily by precipitation methods. Hence, the proposed method is appropriate for the analysis of NAL in pharmaceutical formulations in the presence of above described organic and inorganic compounds.

**Analytical applications**

The proposed FI-CL method was applied to analyse NAL in pharmaceutical samples. The recovery experiments were performed in three ampoules as shown in Table 2 and %recovery was obtained over the range of 96.08–106.06%. In addition, the proposed FI-CL technique for the analyses of NAL in the pharmaceutical injections was validated and the results were compared with a reported spectrophotometric method. For validation, *F*-test and *t*-test were applied on the results of the two methods given in Table 3. Variations in the results of the two methods were not due to any determinate error but were by chance as *F*-calculated value (*F*-test calculated value: 1.69) was lower than *F*-tabulated value (*F*-distributed when *p* = 0.05, *v*₁ and *v*₂ = 2: 19). Similarly, no significant difference was observed between the results of the proposed and reported method that is a good evidence for reproducibility and accuracy of the proposed method. The null hypothesis was retained because paired student’s *t*-test calculated value (Student’s *t*-test value: *t* = 0.40) was obtained lower than *t*-tabulated (*t*-distributed (*p* = 0.05, *v* = 2): 4.30). Therefore, it can be concluded from recovery and validation results that the two methods are not significantly different, and the proposed method has the efficiency to determine NAL in pharmaceutical formulations.

**Kinetic CL reaction curve of Ag(III) complex- Rh-B-Brij-35 with NAL**

In the primary experiments, the kinetic characteristics of the proposed CL reaction based on the oxidation of Rh-B (2.0×10⁻⁵ M) in sulfuric acid (1×10⁻² M) by Ag(III) complex (2.0×10⁻⁴ M) prepared in KOH (1.0×10⁻³ M) and its enhancement by NAL (0.1 mg/L) prepared in Brij-35 (0.01% w/v) in acidic medium were studied by using the batch method. For this purpose, a typical CL intensity against times response curve was utilized to explain the CL system (Fig. 6). The peak of CL intensity appeared within 1 s as the NAL was introduced into the mixture solution of Ag(III) complex-Rh-B-Brij-35 in aqueous sulfuric acid medium and the CL signal took 2.4 s to decreased to the baseline. The kinetic curve revealed that the CL reaction was sensitive enough and so, suitable for the detection of NAL in pharmaceutical formulations.
Possible CL reaction mechanism in acidic medium

In literature, it has been pointed out that when Ag(III) complex is dissolved in aqueous alkaline medium generates monoperiodatoargentate (MPA), which is an active form of the Ag(III) complex during oxidation and making complex with analytes (Reaction-I (R-I)). The generated MPA, in aqueous acidic medium, dissociates and produced silver(III) cation and orthoperiodic acid (H₅IO₆) (R-II). Furthermore, the silver(III) cation oxidizes H₂O molecule and produces superoxide-anion radicals (O₂⁻) and protons (H⁺) (R-III). Electronically excited dimer of oxygen molecule ((O₂)₂*) is generated by the recombination of the generated O₂⁻ (R-IV), which emits CL radiation at 490 nm in acidic medium on de-excitation (R-V).

To investigate the possible CL reaction mechanism, spectrophotometric studies were performed. A UV-visible spectrum for Rh-B solution (2.0×10⁻⁵ M) was obtained in H₂SO₄ (0.01 M) as shown in Fig 7. As a result, maximum absorbance was observed in visible range centered at 557 nm. When the solutions of Rh-B (2.0×10⁻⁵ M) in H₂SO₄ (0.01 M) and Ag(III) complex (2.0×10⁻⁴ M) in KOH (1.0×10⁻³ M) (1.5 mL each) were allowed to mix in a quartz cuvette (3.0 mL), the color of Rh-B (λ_max 557 nm) and Ag(III) complex (λ_max 362 nm) disappeared promptly. These results suggest a redox reaction takes place between Ag(III) complex and Rh-B. Ag(III) acts as a strong oxidizing agent (redox potential of 1.47 V) as shown in reaction-VI (R-VI). Similar observations have also been obtained between the CL reaction of Rh-B and Ce(IV) in aqueous sulfuric acid medium.

The CL emitter in this reaction has been claimed to be a radical, which is one of the oxidized products of Rh-B (Rh-B⁺). The emitter has been further confirmed by infrared, UV-visible and fluorometric studies. When the excited Rh-B radical (Rh-B*⁻*) acting as an intermediate product de-excites, a cold light centered at 425 nm is emitted (R-VII). Rh-B is a fluorophore with wavelength length of 586 nm. Because of the intermolecular collision, the energy may be transferred from (O₂)₂⁻ to Rh-B molecule and got excited electronically (R-VIII), which on de-excitation emits radiation almost at 586 nm (R-IX). The weak CL emission between Ce(IV) and Rh-B in acidic medium has been reported to be further enhanced by synephrine for its determination in fruits, herbal products and biological samples. Similarly, the weak CL emission in the proposed reaction (Rh-B-Ag(III) complex in acidic medium) was enhanced by NAL, which has been reported as a fluorophore with an emission wavelength of 337 nm. Therefore, the energy from the Rh-B*⁻* could be transferred to NAL exhibiting the principle of collisional resonance energy transfer (CRET) producing excited NAL* (R-X). That NAL* emits a CL signal on de-excitation centered at 337 nm (R-XI). Based on the experimental studies and reported literature, the most probable reaction mechanism for the present CL system can be:
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\begin{align*}
\text{[Ag(HIO}_6\text{]}^2^- + 2\text{H}_2\text{O} & \rightarrow \text{Ag(HIO}_6\text{)(OH)}(\text{H}_2\text{O})\text{]}^2^- + \text{H}_2\text{IO}_6^{3-} \quad \text{(R-I)} \\
\text{Ag(HIO}_6\text{)(OH)}(\text{H}_2\text{O})\text{]}^2^- + 5\text{H}^+ & \rightarrow \text{Ag(III)} + \text{H}_5\text{IO}_6 + 2\text{H}_2\text{O} \quad \text{(R-II)} \\
\text{Ag(III)} + 2\text{H}_2\text{O} & \rightarrow \text{Ag(I)} + \text{O}_2^{-} + 4\text{H}^+ \quad \text{(R-III)} \\
2\text{O}_2^{-} & \rightarrow (\text{O}_2\text{)}_2^{*} \quad \text{(R-IV)} \\
(\text{O}_2\text{)}_2^{*} & \rightarrow 2\text{O}_2 + \text{light (490 nm)} \quad \text{(Background CL)} \quad \text{(R-V)} \\
\text{Rh-B + Ag(III)} & \rightarrow \text{Rh-B}^{-}^{*} + \text{Ag(I)} \quad \text{(R-VI)} \\
\text{Rh-B}^{-}^{*} & \rightarrow \text{Rh-B}^{-} + \text{light (425 nm)} \quad \text{(Background CL)} \quad \text{(R-VII)} \\
\text{Rh-B}^{-} + (\text{O}_2\text{)}_2^{*} & \rightarrow \text{Rh-B}^{*} + 2\text{O}_2 \quad \text{(R-VIII)} \\
\text{Rh-B}^{-} & \rightarrow \text{Rh-B} + \text{light (586 nm)} \quad \text{(Background CL)} \quad \text{(R-IX)} \\
\text{Rh-B}^{-}/\text{Rh-B}^{-}/(\text{O}_2\text{)}_2^{*} + \text{NAL} & \rightarrow \text{Rh-B}^{-}/\text{Rh-B}/(\text{O}_2\text{)}_2 + \text{NAL}^{*} \quad \text{(R-X)} \\
\text{NAL}^{*} & \rightarrow \text{NAL} + \text{light (337 nm)} \quad \text{(Strong CL signal)} \quad \text{(R-XI)}
\end{align*}
\]

Fig. 8 reveals the transient CL emission peaks for the reaction between Ag(III) complex-Rh-B-NAL under aqueous sulfuric acid medium. The curve A was achieved when Brij-35 (0.01% w/v) was propelled in all three streams and Ag(III) complex (300 µL, prepared in KOH, 1 mM) was injected into the carrier stream which reveals that Ag(III) complex emits no CL intensity in the absence of NAL or Rh-B compounds. The curve B was achieved when Ag(III) complex (2.0×10^{-4} M, prepared in 1 mM KOH) was propelled in third stream and Rh-B solution (300 µL, 2.0×10^{-5} M in sulfuric acid 10 mM) was injected into the carrier stream, emitted a weak CL response. The curve C was achieved when Ag(III) complex solution and Rh-B solution (2.0×10^{-5} M in sulfuric acid 10 mM) were propelled in their respective streams and NAL (300 µL, prepared in Brij-35, 0.01% w/v) was injected and as a result, an enhancive transient CL peak was achieved. Thus, it can be concluded that the CL intensity generated due to the redox reaction between Ag(III) complex and Rh-B which is further increased by NAL analyte.

**Conclusion**

A novel FI-CL method has been introduced in the present study to determine NAL based on its oxidation with Ag(III) complex sensitized by Rh-B in acidic medium. This method has rapidity, simplicity and reproducibility and improved handling procedure. It has high sensitivity and selectivity in detection of NAL in pharmaceutical formulations with a proposed feasible chemical reaction mechanism. The reagents used in the proposed method are of low-cost, readily available the procedure lacks any tedious sample preparation. Furthermore, the use of Ag(III) complex synthesized in the laboratory is simple and stable in alkaline medium. The results obtained in the proposed method agree well with those of spectrophotometric method.
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Table 1 Analytical characteristics comparison of the proposed method with reported methods for the analysis of NAL in pharmaceutical and biological samples.

| Method       | Sample matrix                                      | Linear range (mg/L) | LOD & LOQ (mg/L) | Sample throughput or analytical time | R²   | Ref. |
|--------------|----------------------------------------------------|---------------------|------------------|--------------------------------------|------|------|
| Spec         | Pharmaceutical & human urine                       | 4.0–80              | 1.02 3.40        | NR                                  | 0.999| 4    |
| Spec method-I| Pharmaceuticals                                     | 1.0–4.5             | 0.217 0.723      | 5 min                               | 0.995| 5    |
| Spec method-II| Pharmaceuticals                                    | 1.0–6.0             | 0.137 0.456      | 5 min                               | 0.997|       |
| Spec         | Pharmaceuticals                                     | 1.0–20              | 0.287 0.869      | NR                                  | 0.997| 6    |
| Spec         | Pharmaceuticals                                     | 2.0–20              | 0.0078 0.226     | 20 min                              | 0.991| 7    |
| Spec         | Ampoules and biological samples                    | 1.6–12.8            | 0.317 1.057      | 10 min                              | 0.999| 8    |
| Spec-Flu     | Pharmaceuticals and biological samples              | 0.5–7.0             | 0.04 0.11        | 31.5 min                            | 0.999| 9    |
| Spec-Flu     | Pharmaceuticals and serum                          | 0.02–0.74           | 0.0037 0.011     | NR                                  | 0.995| 10   |
| Spec-Flu     | Raw material and pharmaceuticals                   | 1.0–12              | 0.175 0.529      | NR                                  | 0.997| 11   |
| Spec-Flu     | Ampoules and biological samples                    | 2.4–8.4             | 0.014 0.047      | 15 min                              | 0.997| 8    |
| Potentiometry| Pharmaceuticals                                     | 0.3939–3939         | 0.3939           | 15 sec                              | 0.998| 12   |
| Voltammetry  | Pharmaceuticals                                     | 3.5×10⁻³–0.03      | 1.898×10⁻⁴       | NR                                  | 0.993| 13   |
| Voltammetry  | Pharmaceuticals and biological samples              | 0.0196–508          | 3.54×10⁻³ NR     | NR                                  | 0.998| 14   |
| Voltammetry  | Biological fluids                                  | 0.0787–196.95      | 0.0275 NR        | NR                                  | 0.990| 15   |
| Voltammetry  | Biological fluids                                  | 0.05–1.25           | 0.013 0.044      | 30 sec                              | 0.997| 16   |
| CZE–UV       | Bulk drugs, pharmaceutical & biological fluids     | 5.0–200             | 0.3 5.0          | 5.19 min                            | 0.999| 17   |
| GC-FID/MS    | Biological fluid                                  | 0.1–10              | 0.05 0.1         | 20.54 min                           | 0.995| 1    |
| GC-MS        | Human hair                                         | 0.18–40             | 0.18             | 18 min                              | 0.995| 18   |
| HPLC-UV      | Injections                                         | 0.5–20              | 0.1 0.5         | 25 min                              | 0.999| 19   |
| HPLC-ECD     | Blood & brain microdialyte                         | 0.025–10            | NR 0.025        | NR                                  | 0.978| 20   |
| HPLC-UV      | Pharmaceuticals                                     | 1.0–15              | 0.243 0.737     | 5 min                               | 0.997| 21   |
| UPLC-MS/MS   | Human plasma                                       | 5.0×10⁻⁵–0.020     | NR 5.0×10⁻⁵     | NR                                  | 0.996| 22   |
| LC-MS/MS     | Human plasma                                       | 5.0×10⁻⁵–0.5       | NR 5.0×10⁻⁴     | 2 min                               | 0.995| 23   |
| FI-CL        | Pharmaceuticals                                     | 0.005–5.0           | 0.001 0.003     | 150 h                               | 0.999| This method |

LOD, limit of detection; LOQ, limit of quantification; R², correlation coefficient; mg/L, parts per million; NR, not reported; Spec, spectrophotometry; Spec-Flu, spectrofluorimetry; CZE-UV, capillary zone electrophoresis-ultraviolet; GC-FID/MS, gas chromatography-flame ionization detector/mass spectrometry; HPLC, high performance liquid chromatography, ECD, electrochemical detector; LC, liquid chromatography; FI-CL, flow injection-chemiluminescence.
Table 2 Recovery of NAL from pharmaceutical ampoules with proposed FI-CL method.

| Sample matrix | Spiked / mg/L | Found / mg/L | Recovery, % | RSD, % (n = 4) |
|---------------|---------------|--------------|-------------|----------------|
| Injection-I   | 0.00          | 0.26         | –           | 2.2            |
|               | 0.25          | 0.53         | 103.92      | 2.1            |
|               | 0.50          | 0.74         | 97.37       | 1.9            |
|               | 0.75          | 0.98         | 97.03       | 1.7            |
| Injection-II  | 0.00          | 0.24         | –           | 2.5            |
|               | 0.25          | 0.48         | 97.96       | 2.4            |
|               | 0.50          | 0.76         | 102.70      | 1.8            |
|               | 0.75          | 1.05         | 106.06      | 1.4            |
| Injection-III | 0.00          | 0.27         | –           | 3.1            |
|               | 0.25          | 0.52         | 100.00      | 2.4            |
|               | 0.50          | 0.77         | 100.00      | 1.7            |
|               | 0.75          | 0.98         | 96.08       | 1.6            |
Table 3 Analysis of NAL in pharmaceutical ampoules and its comparison with a reported spectrophotometric method.

| Sample matrix | Labelled (mg/mL) | Proposed FI-CL method Found (mg/mL) | Spectrophotometric method Found (mg/mL) |
|---------------|------------------|-------------------------------------|-----------------------------------------|
| Injection-I   | 10.00            | 9.95                                | 10.31                                   |
| Injection-II  | 10.00            | 10.21                               | 9.87                                    |
| Injection-III | 10.00            | 9.89                                | 10.13                                   |

*F*-test calculated value: 1.69, *F*-distributed (*p* = 0.05, *v*₁ and *v*₂ = 2) = 19

Student *t*-test value: *t* = 0.40, *t*-distributed (*p* = 0.05, *v* = 2) = 4.30
Fig. 1 Chemical structure of nalbuphine hydrochloride.
Fig. 2 Schematic flow system for the determination of NAL, A, Brij-35 0.01% (w/v) in water; B, Rh-B 2.5×10⁻⁵ M in H₂SO₄ 0.01 M; C, Ag(III) complex 2.0×10⁻⁴ M in KOH 1.0×10⁻³ M; P, peristaltic pump (3.0 mL/min); S, sample loop (300 µL); IV, injection valve; F, flow cell, W, waste, PS, power supply; PMT, photomultiplier tube detector (1000 V); and CR, chart recorder.
Fig. 3 Optimization of (A) sulfuric acid, (B) Ag(III) complex, (C) sensitizers and (D) rhodamine-B concentrations on the detection of NAL.
Fig. 4 Optimization of (A) variable surfactants and (B) Brij-35 concentrations on the detection of NAL.
Fig. 5 CL emission signals obtained for a series of NAL standard solutions injected in triplicate (inset shows calibration curve ranged from 0.005–5.0 mg/L).
Fig. 6 Kinetic curves of Ag(III) complex-Rh-B-NAL. Ag(III) complex (2.0×10^{-4} M prepared in KOH 1.0×10^{-3} M); Rh-B, (2.0×10^{-5} M in sulfuric acid 0.01 M); NAL, (0.1 mg/L prepared in Brij-35, 0.01% w/v).
Fig. 7 UV-visible spectrophotometric spectra (A) Mixture of 1.5 mL of Rh-B solution (2.0×10⁻⁵ M) in H₂SO₄ (0.01 M) and 1.5 mL distilled H₂O (B) Mixture of 1.5 mL of Rh-B solution (2.0×10⁻⁵ M) in H₂SO₄ (0.01 M) and 1.5 mL Ag(III) complex (2.0×10⁻⁴ M) in KOH (1.0×10⁻³ M).
Fig. 8 CL emission peaks for the reaction between Ag(III) complex-Rh-B-NAL under aqueous sulfuric acid medium in flow mode. Curve A, sulfuric acid (0.01 M) and Ag(III) complex (300 µL, 2.0×10^{-4} M prepared in KOH 1.0×10^{-3} M); curve B, Ag(III) complex (2.0×10^{-4} M prepared in KOH 1.0×10^{-3} M) and Rh-B solution (2.0×10^{-5} M in sulfuric acid 0.01 M) and curve C, Ag(III) complex (2.0×10^{-4} M prepared in KOH 1.0×10^{-3} M), Rh-B solution (2.0×10^{-5} M in sulfuric acid 0.01 M) and NAL (300 µL, 1.0 mg/L prepared in Brij-35 0.01% w/v).