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Short communication

Copper based facile, sensitive and low cost colorimetric assay for ampicillin sensing and quantification in nano delivery system

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ABSTRACT

A novel colorimetric assay for quantification of ampicillin has been developed based on Cu-BCA complexation. The developed assay was optimized for determination of drug content in synthesized ampicillin loaded chitosan nanoparticles underlying its importance as a simple, sensitive and economical method for amplicillin quantification developed so far.

1. Introduction

Ampicillin is a well-known antibiotic used for systemic therapy through enteral, parenteral as well as topical administration for gastric/intestinal and other bacterial infections [1,2]. However, owing to short half-life (0.75–1.5 h) of this β lactam antibiotic under *in vivo* conditions [3], there is an urgent need for the fabrication and design of formulations that can provide its sustained and controlled release. For this purpose, a number of drug delivery systems based on polymeric nanoparticles and matrices have been reported for entrapping antimicrobial drugs serving as protective measure for healthy cells as well as the fragile drugs [4].

The drug entrapment efficiency and loading content evaluation have generally been subject of challenge for synthesizing ampicillin impregnated systems for drug loading due to lack of established method for quantification of this drug. In this context, UV-visible analysis has been generally used to measure the absorbance and analyze the amount of ampicillin trihydrate [5,6], However, this method suffers from a serious drawback as the ampicillin molecule does not absorb strongly in UV–Vis range. Apart from this, chemiluminescence, fluorescence, liquid chromatography, and electrochemical based methods have been reported for determination of different β- lactam antibiotics [7]. In particular, the chemiluminescence and fluorescence based techniques require derivatization with various expensive chemiluminescent and fluorescent agents, respectively [8,9] whereas HPLC requires sophisticated instrumentation and time consumption. A quantitative method using FT-IR has been recently proposed for analysis of ampicillin sodium salt in powder for injection but it displays very low sensitivity [10]. As none of the available methods employ a sensitive and economical approach for estimating the amount of ampicillin in a given system, herein we report a simple and facile colorimetric method for the estimation of this therapeutically important antimicrobial drug and its application in drug determination in chitosan nanoparticle system.

2. Materials and methods

2.1. Materials

All the reagents used were of analytical grade and were used without further purification except otherwise mentioned. Bicinchoninic acid disodium salt hydrate (≥98%,HPLC) (BCA) and Chitosan (low molecular weight)(Mol wt 50,000–190,000 Da), deacetylated at 75–85%, and chitosan (high molecular weight), deacetylated at >75%, sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (India). Sodium carbonate,

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sodium tartarate, sodium hydroxide, sodium bicarbonate, cupric sulphate·5H_2O were purchased from Fisher Scientific (India). Tris base was purchased from CDH (India). Hydrochloric acid and Acetic acid (99.8%) was purchased from Avra Synthesis Pvt. Ltd. (India). Ampicillin sodium salt (molecular weight 371.4 g/mol), Kanamycin and streptomycin were purchased from Himedia, Mumbai, India. Deionized water obtained from Millipore purification system with a resistivity of 18.2 μΩ was used in each experiment.

2.2. Design of assay and preparation of calibration curve

For preparing buffered BCA solution, 1 g BCA, 2 g sodium carbonate, 0.16 g sodium tartarate, 0.4 g sodium hydroxide and 0.95 g sodium bicarbonate were dissolved in 100 ml of distilled water and its pH was set to 11.25 using 10 M sodium hydroxide solution. Another solution was prepared by dissolving 0.4 g of cupric sulphate·5H_2O in 10 ml of DI water separately. Standard working solution (SWS) was finally prepared prior to use by mixing 100 volumes of buffered BCA solution with 2 volumes of copper sulphate solution which resulted in green coloured solution.

For the preparation of calibration curve, ampicillin solution of varying concentrations (0.062–1 mg/ml) were prepared in triplicate. 20 μl of ampicillin solution (or unknown sample) was incubated with 1 ml of standard working solution for 25 minutes at 60^{O} C using incubator shaker to obtain purple coloured solutions of varying intensity. The UV–visible absorption spectra were recorded using spectrophotometer for various solutions. Calibration curve was plotted using absorption at 564 nm to obtain equation of a logarithmic progression.

2.3. Preparation of chitosan solution

Commercially available chitosan is not readily soluble in water at neutral pH. So, solution of both type of chitosan (LMW and HMW) was prepared by dissolving in 0.1% acetic acid at room temperature. For complete dissolution, few more drops of acetic acid were added and solution was filtered to remove any particulate matter. The pH of the resultant solution was the adjusted to 5.5 using standard base.

2.4. Synthesis of low molecular weight and high molecular weight chitosan nanoparticles

In this study, modified ionic gelation method was used for the synthesis of uniform and spherical chitosan nanoparticles employing sodium tripolyphosphate as ionic cross linker. Briefly, 2 ml of 0.1% of TPP solution was added dropwise to 5 ml of 1 mg/ml chitosan solution containing 0.01% Kolliphor as stabilizer, under constant magnetic stirring at 800 rpm. After 30 min of stirring, the solution was sonicated for 15 min using sonicator bath. The nanoparticles were lyophilized to get white powder form in each case.

2.5. Synthesis of ampicillin loaded chitosan nanoparticles

Ampicillin loaded nanoparticles were prepared by using similar protocol as chitosan nanoparticles. 1 ml of 1 mg/ml solution of ampicillin was added to previously prepared chitosan solution followed by the similar steps as above. The formed nanoparticles were purified by cycles of centrifugation and re-dispersion into deionized water. The supernatant was collected for unloaded drug estimation. The purified nanoparticles were lyophilized using freeze vacuum drying.

2.6. Determination of encapsulation efficiency and drug loading

Encapsulation efficiency and total amount of drug loaded into the nanoparticles was determined using developed Cu-BCA colorimetric assay. Briefly, the different nanoparticles solutions were centrifuged at 8000 rpm. The supernatant and pellet were collected separately. The supernatant so obtained was incubated with SWS at 60 ^{O}C and its absorption at 564 nm was recorded. Using equation obtained from calibration curve, amount of free ampicillin in supernatant was calculated.

For calculating encapsulation efficiency and drug loading, the following equation was used:

$$\text{Encapsulation efficiency, (EE)} = \frac{\text{Total amount of ampicillin added} - \text{Amount of ampicillin present in supernatant}}{\text{Total amount of ampicillin added}} \times 100 \quad (1)$$

$$\text{Drug loading, (DL)} = \frac{\text{Total amount of ampicillin added} - \text{Amount of ampicillin presentin supernatant}(\mu g)}{\text{Amount of polymer (mg)}} \quad (2)$$

2.7. In vitro release studies

The release of drug from the nanoparticles was studied in three different buffer systems namely sodium hypophosphate – acetic acid (pH 5), 1x PBS (pH 7.2) and Tris–HCl (pH 9) for providing different pH conditions. Briefly, 15 mg of lyophilized nanoparticles were dispersed in 15 ml of each buffer separately and were incubated at 37 ± 1°C with rotation speed of 100 rpm (Incubator Shaker, Lyzer, India). At predetermined time intervals, 1 ml aliquot from each system was removed and was replaced with fresh buffer. The removed nanoparticle aliquot was centrifuged at 8000 rpm (Laby microcentrifuge machine, India) to pellet down nanoparticles and amount of drug released was determined in supernatant using colorimetric assay employing UV-spectrophotometer at 563 nm and cumulative percent release was calculated.

2.8. Interference studies

20 μl each of 0.1 mM solution of ampicillin, streptomycin and kanamycin were incubated with 1 ml BCA-Cu complex solution for 25 min at 60 °C and absorption of the resultant solutions was analyzed spectrophotometrically.

3. Results and discussion

There are number of metal based colorimetric assays available for detection of different biological molecules [11,12]. Amongst the transition metals, copper in particular, is known to form coloured coordination complexes with variety of ligands. The copper ions in different oxidation state can bind to a number of ligands and show intense colour at specific temperature and pH conditions [13]. Due to this property of spontaneous oxidation/reduction and display of distinct colour change in the process, copper based moieties have been used to develop a number of colorimetric assays. Most common examples are Biuret test [14] and Lowry assay [15] that have long been used for the detection of proteins in a solution. Bicinchoninic Acid (BCA) assay [16] for detection and quantification of total protein employs BCA which is structurally comprised of two carboxylated quinoline rings which are known to bind with Cu^{2+} to give a weak Cu^{2+}-BCA complex. This complex which has been widely exploited for detection and determination of proteins and reducing sugars.