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Development and Validation of a Reversed-Phase HPLC Method for In-Vitro Loading and Release Analysis of Paclitaxel Coated Stent

Jaynish Tailor, Ankur Raval, Chhaya Engineer, Haresh Kotadia and Devesh Kothwala

Research & Development Division,
Sahajanand Medical Technologies Pvt. Ltd., Surat 395 003, India

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A simple, rapid and sensitive high-performance liquid chromatographic method is described for determination of Paclitaxel (PCL) from coated stent surface using a reverse phase X-terra C_{18} (5 ìm) column at 227 nm and using acetonitrile, methanol and water in the ratio of 60:5:35 (v/v) as a mobile phase following single-step extraction from Phosphate buffer saline (PBS) pH 7.4 with dichloromethane. The assay was validated against the classical criteria and was applied to detect paclitaxel content as well as release amount from the stent surface. Sink condition of paclitaxel coated stent was maintained in PBS throughout the experiments by different criteria. It was maintained by modifying the release medium such as methanolic PBS, PBS containing DENA (N, N- Diethylnicotinamide), and 0.1% sodium azide in PBS. With the "sampling" technique, drug-loaded stent was introduced into a vessel, and release was monitored over a period of time by analyzing aliquots of release medium. In the use of agitation by means of orbital shaking incubator, the sampling seems to be easier than the continuous flow method. In the "continuous flow" technique, media is continuously circulated through a cell containing drug-loaded stent followed by analysis. The extraction method achieves a chemical separation of drug from the release media by use of a separating funnel. With all these methods, the setup and sampling techniques seem to influence in vitro release. © *Society for Biomaterials and Artificial Organs (India), 20071205-20.*

Introduction

The concept of the stent grew directly out of interventional cardiologists' experience with angioplasty balloons in the first decade of use (1977-87). Sometimes, the wall of the coronary artery became weakened after balloon dilatation. Although the artery opened successfully using a balloon, in a small percentage of cases, the artery would collapse after the balloon was deflated. Since there was no interventional "fix" available, the only option for this patient was emergency bypass graft surgery to repair the problem. One such device was the stent, a metal tube or "scaffold" that was inserted after balloon angioplasty. More recently a normal metal stent that has been coated with a pharmacologic agent (drug) that is known to interfere with the process of restenosis (reblocking). Restenosis has a number of causes; it is a very complex process and the solution to its prevention is equally complex. However, in the data gathered so far, the drug-eluting stent has been extremely successful in reducing restenosis from the 20-30% range to single digits.[1] Paclitaxel is an anticancer drug which helps to prevent restenosis in the artery after stenting. Paclitaxel (PCL), a diterpene amide derived from Pacific yew tree, the most important anticancer agent for the past 18 years. Paclitaxel received FDA approval for the treatment of ovarian cancer in Dec. 1992, metastatic breast cancer in April 1994 and lung cancer.

Stent implantation has become the major method of percutaneous myocardial

revascularization. However, in-stent restenosis continues to limit the long-term success of this approach. The concept of local drug delivery via coated stents, couples the biological and mechanical solutions necessary to maximize the angiographic results and facilitate the recovery of the vessel from the injury caused by the stent implantation. At the same time local drug delivery using a drug eluting stent offers the advantage of allowing high local concentrations of drug at the treatment site while minimizing systemic toxic effects. Biodegradable polymers have been used in controlled drug delivery for many years. These biodegradable polymers degrade within the body as a result of natural biological processes, eliminating the need to remove a drug delivery system after release of the active agent has been completed. The reported mechanism of erosion, which controls the release of drugs from cardiovascular stents, is insufficiently investigated. The current research work aims at probing the mechanism of paclitaxel drug release from multilayer drug-polymer coated stent as well as HPLC method validation for detection of paclitaxel. The surface morphology of drug-coated stent was observed using scanning electron microscopy before and after incubation in phosphate buffer saline solution (pH 7.4) at 37°C to understand the in-vitro drug release mechanism. High pressure liquid chromatography was used to explore the drug release kinetics. [9]

Materials and Methods

The SS 316 LVM 16-mm long MATRIX™ stents (Sahajanand Medical Technologies, India) coated with Paclitaxel drug (Infinnium™) were used in the study. Paclitaxel drug was obtained from Bioxel Pharma Inc., Canada and used without further purification. Blend of polymers; 50/50 Poly DL-Lactide-co-Glycolide, 75/25 Poly L-Lactide-co-Caprolactone (Alkermes Inc., USA), Poly L-Lactide (Purac Inc., USA) having inherent viscosity (IV) 0.60 dL/g, 1.2 dL/g and 1.63 dL/g respectively were used as carriers for drug. Poly vinyl pyrrolidone (PVP K-90/D) was procured from ISP Technologies Inc., Wayne, NJ, USA. The typical molecular weight for PVP K-90/D was 1300000 Da. HPLC grade solvents such as Dichloromethane (DCM), Acetonitrile (CH_3CN), methanol (CH_3OH) were purchased from Qualigens fine chemicals (Division of Glaxo SmithKline, India) and water was purchased from Merck specialties Pvt. Ltd, India. Other ExcelaR grade chemicals such as Dihydrogen sodium orthophosphate ($Na_2HPO_{4)}$, Potassium di hydrogen orthophosphate (KH_2PO_4) and Sodium Chloride (NaCl) were also purchased from Qualigens fine chemicals (Division of Glaxo SmithKline, India).

HPLC assay

Analysis was performed on HPLC-LC-2010 AHT [Shimadzu]. The analytical column was X-Terra C-18 (250mm × 4.6 mm), particle size 5µm from Waters. The system was equipped with Autosampler and column oven set at room temperature. The mobile phase was a mixture of Acetonitrile (60%), Methanol (5%) and Water (35%) used after filtration through 0.22 µm membrane filter and degassed by ultrasonicator using frequency 40 KHz. Injection volume was 20µl. The flow rate was 1.0 ml/min which led to pressure of 90-120 kgf/cm^2. PCL was detected by UV absorption at 227 nm at retension time of approximately 5.2 ±1.0 min. PCL concentration was calculated by comparing the peak area of standard and sample. Content of paclitaxel loaded on the stent was dissolved in 10 ml of mobile phase and was analyzed by HPLC technique and was compared to standard to quantify the content.

Sampling technique for drug release

Stents were evaluated for in-vitro paclitaxel release kinetics from biodegradable polymer matrix over 48 days in phosphate buffer saline (PBS) solution (pH 7.4) at 37°C with constant agitation at 55 rpm. PBS was replaced with fresh PBS at intermittent interval from their release vial and analyzed for amount of paclitaxel release.

Drug release is monitored at intermittent intervals by extracting the drug form PBS. The volume of supernatant withdrawn depends on drug solubility and stability, assay sensitivity, and maintenance of sink conditions. For poorly water-soluble drugs, such as paclitaxel, all of the release media (10 ml) was withdrawn at each

analysis followed by replacement with the exact volume sampled exact volume sampled (7).

Three stents were evaluated for paclitaxel content and two stents for in-vitro paclitaxel release kinetics from biodegradable polymer matrix over 48 days in phosphate buffer saline (PBS) solution (pH 7.4) at 37°C with constant agitation at 55 rpm. All the stents were removed at intermittent interval from their release vials and analyzed for amount of paclitaxel release in PBS. Scanning electron microscopy (SEM) was done to analyze the coating morphology and degradation mechanism.

Use of orbital shaking incubator

Drug coated stent was kept in 10ml of PBS and incubated at 37ºC temperature with 60(±5) RPM by means of Orbital shaking incubator. Aliquots were withdrawn on intermittently followed by replacement of fresh PBS. The process of taking aliquots continued for 48 days.

Continuous flow method

Constant flow (CF) of media is achieved by using a peristaltic pump. With the CF setups using peristaltic pumps illustrated in fig. 1, the buffer may be recirculated [7] or the fresh buffer may be pumped constantly through the vial containing drug coated stent. We used a peristaltic pump to achieve constant flow. A peristaltic pump connected to buffer reservoir placed in a circulating water bath at 37°C to assess the in vitro release of drug. Selection of a flow rate seems to depend on the type of pump used to study in vitro release [4].

In most cases, a lower flow rate resulted in incomplete release probably because of slower rates of hydration and dissolution of the polymer and drug, respectively. Conversely, cumulative release greater than 85% was obtained with higher flow rates. Hydration of the polymer matrix is the most important factor governing the release from drug-polymeric matrices systems. Once the polymer is hydrated, drug release occurs as a result of a combination of diffusional and erosional processes. A lower flow rate led to slower hydration of the polymer matrix [4].

Extraction of Paclitaxel from PBS

PBS in which drug has been released was transferred into the separating funnel. It was extracted by means of Dichloro methane (DCM) solvent by vigorous shaking. Both phases were allowed to be separated. Organic phase was collected the and further DCM was added to extract the remaining drug from PBS. These two volumes of DCM extract were mixed and evaporated all the portion of DCM and drug remain as in the form of residue. The drug residue was dissolved in mobile phase. 20µl of this solution was injected in HPLC for analysis.

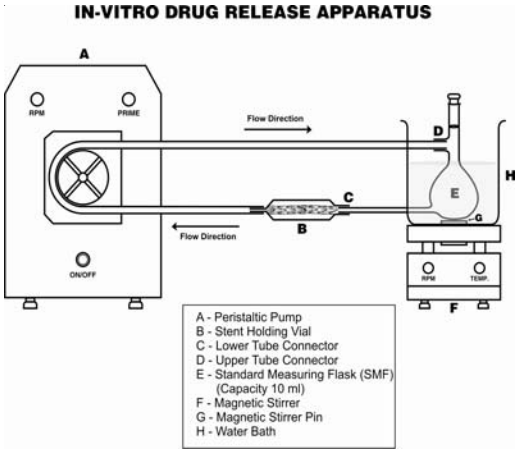

Fig 1: Commercial apparatus for continuous flow method using peristaltic pump with recirculating buffer solution

Probable causes of decreased drug release and its preventions

Initially In-vitro drug release was performed in the Phosphate Buffer Saline (PBS) having pH 7.4 similar to pH of human blood. Incomplete drug release was a major crisis for invitro drug release study.

As per the experiments done and literature reports the probable causes for such kind of drug release could be following.

Due to lower solubility of drug in PBS, sink condition is not maintained in the release medium. Therefore it is wise to employ sink conditions

during in vitro testing. In the event that a small volume of media can be used (based on the method employed and assay sensitivity), total media replacement may be used to ensure drug solubility, maintain sink conditions, and prevent accumulation of polymer degradation products.

Drug elution from the stent can occur over an extended period of time depending on solubility of the drug and the non-drug matrix. It may be necessary to use hydro-alcoholic media to accelerate the dissolution rate. This option can be used if aqueous medium are not adequate to fully elute the drug to >80% or an asymptote. Recommended alcohols are methanol, ethanol and isopropyl alcohol [2]. For Paclitaxel Buffer: Methanol (90:10) has been used as the release medium [5].

Microbial growth in PBS

For release study samples are kept in PBS (pH 7.4) and incubated at 37°C. Microbial growth was observed in PBS after some period of time. As PBS is an aqueous medium provides ideal conditions for microbial contamination. So it was necessary to add preservatives into PBS. So PBS was modified by introducing 0.1% sodium azide to prevent microbial contamination.

Drug adsorption property of glassware

On account of a research article it was predicted that due to long time contact with glass, some amount of drug gets absorbed by the glass surface. The stability of paclitaxel in aqueous solution and apparent binding of drug onto the walls of containers made of different materials. Glass and polypropylene vials are believed to absorb paclitaxel [3]. This is why none of the published in-vitro release data on paclitaxel report 100% release.

Surface contact to glassware

During extraction process of drug form PBS, it comes into the contact with glass wares frequently. So it can be assumed that drug can be adsorbed to the glass surface and drug loss might be observed in final results. After completion of extraction process the funnel is rinsed with DCM to collect surface adhered drug. This content is mixed to previously extracted volumes of DCM to minimize drug loss. Another way to prevent such type of loss whatever the drug released in PBS should be directly injected. For such type of experiment release media can be modified by introducing such solvent in which drug is soluble.

N, N-Diethylnicotinamide (DENA) has been identified as an excellent hydrotropic agent for paclitaxel. The aqueous solubility of paclitaxel was increased by several orders of magnitude in the presence of DENA. Because of such a high hydrotropic property, DENA was used as a release medium providing a sink condition for the release of paclitaxel from drug polymer matrices. So by introducing DENA extraction step could be deleted and we can analyze the drug release content by injecting such release medium directly [5].

HPLC Method Validation

The validation of this method was performed in our lab in accordance with the regulatory guidelines of FDA

Accuracy and precision

Accuracy and precision were assessed using 10µg/ml and 30µg/ml concentration of PCL respectively. Each level was assayed in six replicate in a single day. At each recommended level studied, replicate samples are evaluated. The %RSD of the replicates found to be less than 2.0%. For accurate and précised method %RSD must be less than 2.0 %

Stability

The sample was analyzed at different time intervals to assess any change in peak area of drug whilst the solution is protected from physical and chemical stress. For this study drug solution of 20µg/ml was kept in refrigerated condition and analyzed after 24 hrs.

Linearity

The linear range of delectability that obeys Lambert Beer's Law is dependent on the compound analyzed and detector used.

Linearity study was performed by single measurement at several analyte concentrations in increasing order from the limit of quantification. The obtained data was treated statistically for calculation of regression coefficient (R^2 Value). Under most circumstances regression coefficient R^2 is e" 0.999.

Limits of detection and quantification

The limit of detection was determined by injecting mobile phase initially and then PCL standard. The limit of detection was calculated by comparing the signal to noise ratio of blank and area of standard at retention time of analyte. The area of standard should be at least three times the noise level.

The limit of quantification is that concentration of standard at which peak area of analyte is ten times that of noise level of blank mobile phase at the retention time.

Detection limit is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental condition. Quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Results and Discussion

Bioerodable Controlled Drug Release Mechanism

In chemically controlled systems, chemical control can be achieved using bioerodible or pendant chains. The rationale for using biodegradable systems is that the bioerodible devices are eventually absorbed by the body and thus need not be removed surgically. Polymer bioerosion can be defined as the conversion of a material that is insoluble in water into one that is water-soluble. In a bioerodible system the drug is ideally distributed uniformly throughout a polymer in the same way as in monolithic systems. As the polymer surrounding the drug is eroded, the drug escapes (Figure 2). In a pendant chain system, the drug is covalently bound to the polymer and is released by bond scission owing to water or enzymes. In solvent-activated controlled systems, the active agent is dissolved or dispersed within a polymeric matrix and is not able to diffuse through that matrix. In one type of solvent-controlled system, as the environmental fluid (e.g., water) penetrates the matrix, the polymer swells and its glass transition temperature is lowered below the environmental (host) temperature. Thus, the swollen polymer is in a rubbery state and al-

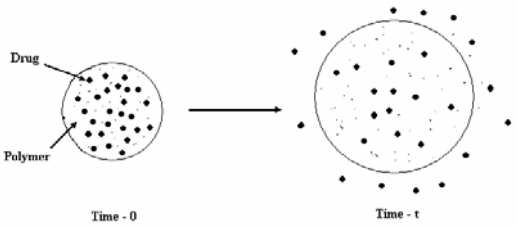

Fig 2: Schematic representation of drug release form drug-polymer matrix

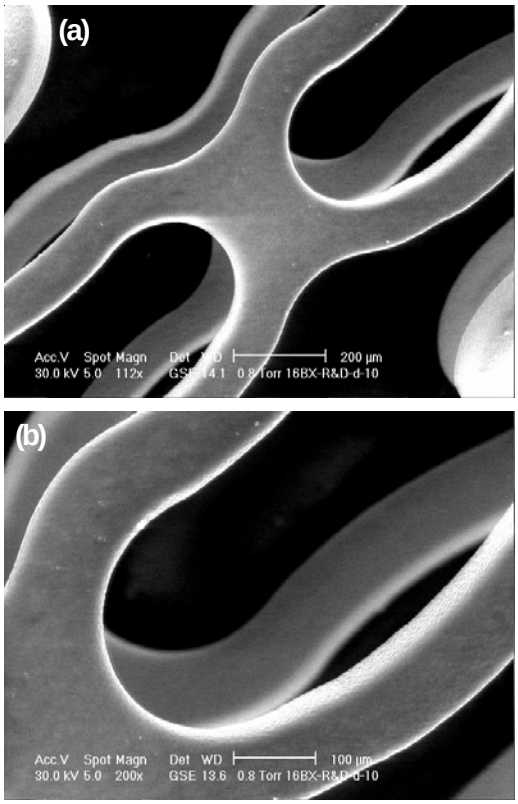

Fig 3: (a) & (b): Scanning Electron Micrograph of drug-polymer coated stent

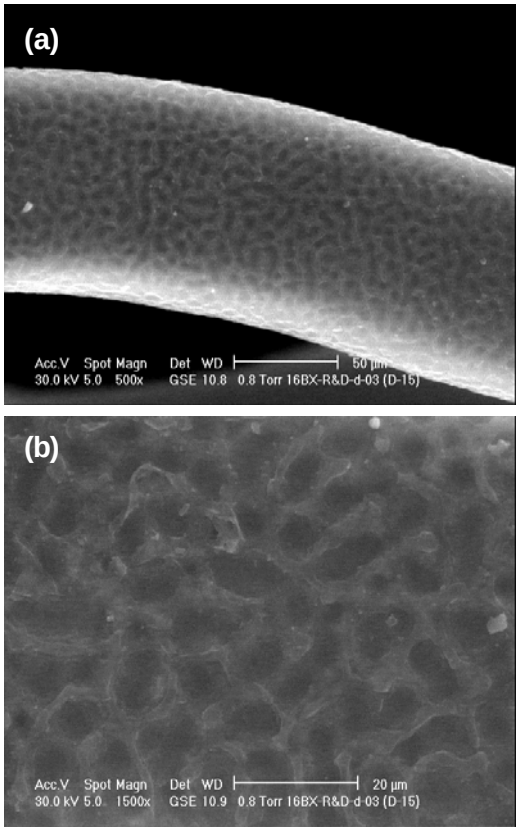

Fig 4: (a) & (b): Scanning Electron Micrograph of drug-polymer coated stent after incubating in PBS at 37°C for 15 days

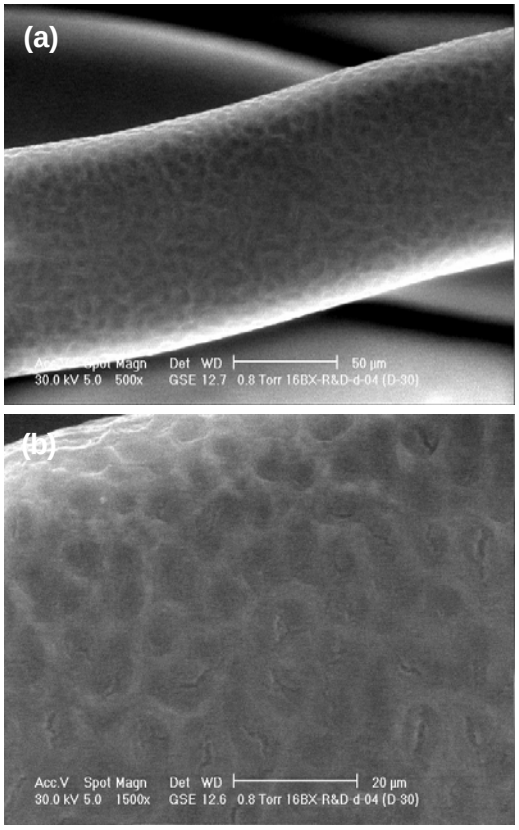

Fig 5: (a) & (b): Scanning Electron Micrograph of drug-polymer coated stent after incubating in PBS at 37°C for 30 days

lows the drug contained within to diffuse through the encapsulant.

Representative SEM images of the surface of drug-polymer coated stents can be seen in figure. Morphology of the drug-polymer coated surface reveals a smooth appearance. [Figure 3 (a) & (b)]

The surface of the coated stent was found to be free from any irregularities such as cracking, flacking and delamination. The SEM image of cardiovascular stent in Figure 4 and figure 5 were obtained after 15 days and 30 days of incubation in PBS at 37°C respectively. Small voids observed on the surface, were regions previously occupied by paclitaxel drug particles that were released from the coating. These SEM images are evidence for in-vitro release of paclitaxel from the drug-polymer matrix. This indicated that release of drug is via dissolution of the paclitaxel particles from the surface of the coated stents and rapid bulk erosion of the polymer.

Comparative release result of PCL with and without extraction

Due to less solubility of drug in PBS the lower amount of drug was analyzed by the aid of the HPLC technique. From the experiments performed it was concluded that the drug amount released in PBS is not the actual amount because after extracting the drug in DCM the amount release was found more than the release analyzed in PBS.

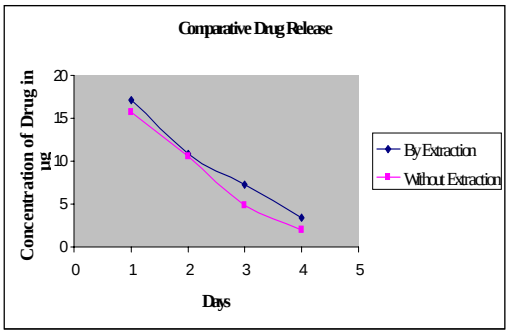

Fig 6: Comparative release of PCL for four days with and without extraction

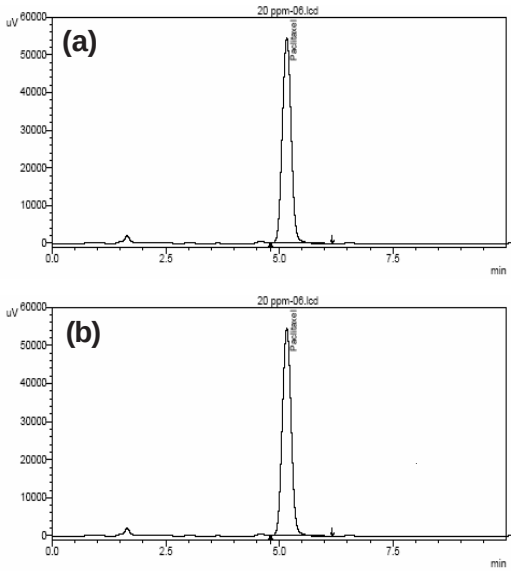

Fig 7: (a) & (b): Typical chromatograms of (A) 20 ppm of PCL at initial phase, (B) 20ppm of PCL after 24 hrs

The degradation rate of Paclitaxel in 2 M DENA was not larger than that in PBS, suggesting that the use of DENA in a release medium did not increase the degradation rate of Paclitaxel. The 2 M DENA aqueous solution was successfully used as a release medium for *in vitro* Paclitaxel release from stents of various formulations [4, 5].

Stability

The 20µg/ml of PCL solution was analyzed initially and after 24hrs. No significant difference

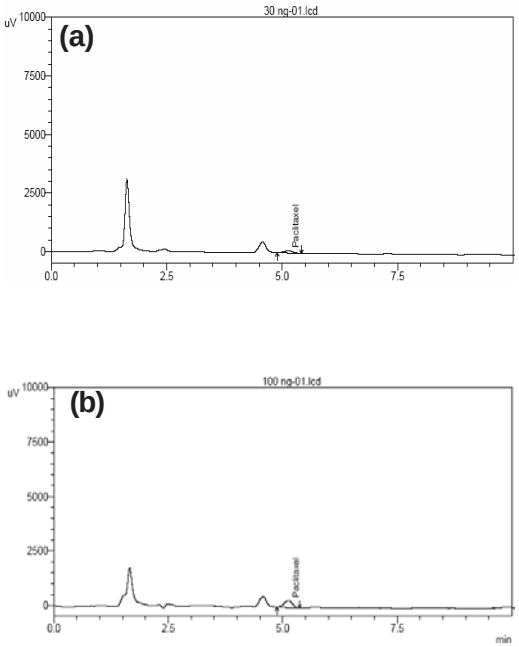

Fig 8: (a) & (b): Typical chromatograms of (A) detection limit for PCL and (B) quantification limit for PCL

Table 1: Tabular interpretation of stability of analyte

Condition	Area
Initial Area	734968
Area after 24 hrs.	738713

in the area of peak was observed (fig 7 & table 1.)

Accuracy and precision

The % RSD (Relative standard deviation) values for the peak area of 10µg/ml and 30µg/ml concentration of PCL were found to be 0.147 and 0.114 respectively.

Linearity

The regression coefficient was found to be 0.9976 for Paclitaxel within the concentration range 1 µg/ml to 50 µg/ml. The proposed method showed linearity between 1-50 µg/ml.

Limits of detection and quantification

The limit off detection for PCL was found to be 30ng which was three times the noise level [Fig. 8(a)]. The quantification limit for PCL by consequently analysis was found to be 100 ng [Fig. 8 (b)].

Conclusion

Present research work investigates the drug release mechanism from multilayered drug polymer coated cardiovascular stent using SEM and HPLC. It was observed that release of drug was due to intake of fluid from simulated biological environment (PBS). The swelling increases the aqueous solvent content within the formulation as well as the polymer mesh size, enabling the drug to diffuse through the swollen network into the external environment. This review also illustrates that sampling with the use of agitation by orbital shaking incubator method easier than the continuous flow method by means of peristaltic pump. Although in vitro release tests can be used as an indicator of in vivo performance for cardiovascular stent systems and also as a quality-control tool. Future research with such a controlled drug delivery system should focus on developing a release method that would be applicable to wide spectrum of drug molecules and polymers.

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