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# APPLICATION OF NANOTECHNOLOGY IN PHARMACEUTICAL PRODUCT DEVELOPMENT

**A thesis submitted in partial fulfilment for the Degree of Master in Science in Pharmaceutical Analysis at the University of Strathclyde**

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**ABSTRACT**

Bioavailability (with high permeability and solubility) is important for effective therapeutic drugs. Nevertheless, in drug research, many drug molecules are of low permeability and solubility. Accordingly, in order to prolong the release and boost the bioavailability of these drug substances, new methods are required. A nanoparticle is a new solution to frame drug agents into nanoparticles. Two drugs fragments like furosemide (diuretic) suit to the BCS class IV and Amlodipine Besylate (calcium channel blocker) suit to the BCS class II in this study were co-encapsulated into PLGA nanoparticles by PVA as a surfactant. Normal particle dimension of these nanoparticles was found to be 275 nm with -12 mV zeta potential value. In house HPLC technique was developed for simultaneous estimation of these two molecules using CAN 5 C18-AR HPLC column with Phosphate buffer: CAN (62:38) as a mobile phase (1.0ml/min flow rate, 220nm detection) for entrapment efficacy study. Normal entrapment efficiency of Amlodipine and Furosemide were found to be 40% and 58%. Additionally, in vitro dissolution study was conducted in the phosphate buffer pH 7.4, and it was witnessed that 6% and 10% amlodipine and furosemide were released after 24 hours at room temperature condition at 100 rmp. From these results, it was concluded that these two molecules could be co-encapsulated in PLGA nanoparticles and below 15% relief of these two molecules after co-encapsulation in the PLGA nanoparticles showed the controlled release arrangement.

## 1. Introduction:

The present trend in technology is now showcasing the new emerging class of therapeutics in medicine using the Nano technology platform, which is an exciting area of research with developments which will improve the success of the efficient drug delivery systems and other treatment options. Nanotechnology has facilitated the manufacture of complex structures in a very small size such as "nanometre" in drug development. However, to use the potential of this technology in pharmaceutical drug development, attention is needed to safety and toxicological studies, as, for some formulations, evaluation is needed to prevent undesired effects and hazards (Wim &Paul 2008).

### 1.1 Drug delivery routes:

Drug delivery route is the path by which a drug is administered into the body. The route and method by which a drug is delivered can have a significant effect on its efficacy (Wim &Paul 2008). The release of the drug is targeted to specific areas or specific sites of the body. The choice of a delivery route is based on many factors like patient acceptability, characteristics of the drug (its metabolism rate, physical and chemical properties) etc. Various types of drug delivery routes exist like pulmonary, transdermal, parenteral routes (intravenous, intramuscular, subcutaneous), trans-tissue, per-oral (non-invasive) trans-mucosal, topical (skin), trans-dermal patches, nasal sprays, auto-injector pens and other inhalation routes.

#### 1.1.1 Oral route:

Oral route of drug administration is the process by which drugs are delivered by mouth and this mode of drug delivery is most preferred, as it is convenient, patient friendly, cost – effective (Yellela, 2010). Drugs administered orally are effective across a broad range of molecules and can be formulated in numerous dosages and in conjugated forms using nanoparticles for selective binding (Hussain et al. 1997) (Mittal, 2007). The availability of drugs administered through oral route depends on factors like dissolution rate, solubility, drug-permeability etc. (Merisko & Liversidge 2008) Drugs with right pharmacokinetic / pharmaco-dynamic profile are readily deliverable orally.

#### 1.1.2 Advantages and disadvantages of oral route administration:

Oral route of drug delivery is most commonly used due to many advantages it offers ; ease of administration, high patient compliance, reduced inter-subject variability, being cost-effective, rapid onset of action, practically safe and sterile etc. (Sanjay & Shringi 2003) .Disadvantage of this mode of drug delivery include the low bioavailability of some specific drugs (with low solubility and permeability). Novel technologies are being developed to

address these challenges. However, this mode is not suitable for large molecules like proteins, thus limiting the potential of this route of drug administration. Some drugs delivered through this route get degraded by high acid content and digestive enzymes, there is improper absorption of drugs in the epithelial membrane and some drugs change into different forms at varied levels of pH, thus affecting the absorption rate.

**1.2 BCS classification of drugs:**

Absorption of any drug via oral route depends mainly on two factors. One of the factors is the dissolution rate and the other factor is permeability across GI tract. These factors are the foundation of BCS. The BCS (biopharmaceutic classification system) is a drug substance based on its PH dependent aqueous solubility and intestinal permeability (Amidon et al, 1995).

**Class I: No solubility, permeability issues:**

Rapid and complete absorption of the drug takes place due to suitable absorption, permeability properties of this class of drugs. Dissolution is the rate limiting step here, as a high dissolution rate allows rapid gastric emptying which further becomes the rate determining step, so a medium which reflects the gastric conditions should be used. The bioavailability of this class of drugs is usually complete due to favourable absorption and permeability properties (Yasir et al. 2010).

**Class II: Low solubility and high permeability issues:**

Dissolution needs to be complete before the dosage form transits from the site of action which otherwise would lead to poor bioavailability of the drug (Merisko & Liversidge 2008).In case of poorly soluble drugs, where dissolution is the rate-limiting step, drug delivery using polymeric nanoparticles is the best solution, as the dissolution rate gets enhanced due to the availability of soluble form of the drug and elimination of other factors which hamper the absorption of the drug (Merisko & Liversidge 2008).

As, rate at which dissolution occurs is directly proportional to the surface area, when the active compound is reduced to nanometre particle size, the surface - area-to -volume ratio gets increased to a tremendous extent thereby enhancing the bioavailability of the molecule as its absorption capacity increases (Merisko & Liversidge 2008). Thus, nano sizing can be used for poorly soluble drugs to increase the dissolution rate thereby improving their bioavailability by delivering the contents at intracellular sites.

**Class III: Low solubility and low permeability:**

Polymeric nanoparticles enhance delivery to target tissues due to increased drug permeability or absorption, minimize the side effects and allow sustained drug release over

prolonged period of time, also reducing the dosing frequency and improving patient compliance. Nano-carriers are an ultimate solution because of their capacity to accumulate on the sites of action like inflammatory sites due to their enhanced permeability and retention properties, thus facilitating supply of drug for a prolonged duration at the site of action.

**Class IV: High soluble and low permeability:**

Drug dissolution is the process by which molecules present in the solid phase enter into a solution phase and get absorbed (Mohanchandran et al. 2010). Molecules must possess some characteristics like hydrophobicity or lipophilic nature and should interact with other biological molecules, either through dissolution or permeability, so as to retain their capacity to dissolve (Merisko & Liversidge 2008). As poorly water-soluble molecules have weak to moderate physicochemical properties, low bioavailability; nano carrier approach can be used to improve solubility, permeability, bioavailability, equivalence, optimal dosing and at the same time for retaining the biological activity of the drug.

**1.3 Nano particle carrier system as a solution for limitations due to other compounds:**

Nanoparticles exhibit some novel properties and multi-functions like small size, enhanced saturation solubility, increased dissolution velocity, customized surface, prolonged shelf-stability and capacity to function as efficient carriers when compared with other compounds (Wim &Paul 2008). They also increase the bioavailability of drugs, improve solubility, target a number of diverse cell types - so are a solution to limitations of micro-particles and other larger micro-particles (Merisko & Liversidge 2008). Drugs are encapsulated as nanospheres / nano capsules onto a nano-matrix or nanoparticle carriers (Chavanpatil et al. 2007) .These carriers facilitate targeted delivery of drug to the site of action. Nanoparticles enclosed by polymeric membrane can be used to achieve both controlled drug release for prolonged duration at the site of action through modulation of the polymer characteristics.

**1.3.1 Application of Nanotechnology – direct nano sizing and incorporation into polymeric and lipidic nanoparticles:**

In Nano sizing, desired drugs are reduced to sub-micron or nano-meter particle size, by application of Nanotechnology (Yellela, 2010). Dissolution rate of the drug improves through nano sizing, in which mechanical attrition or homogenization process is used to render large crystalline particles into nanoparticles (Yellela, 2010). In the next step, these particles are stabilized and then formulated to a desired combination, so as to retain their original characteristics with the help of surfactants ((Sahana et al. 2008) or polymers in nano-suspensions (like poly (lactic acid), poly (lactide-co-glycolide) [PLGA] (Bala et al.2004)

(Mohamed & van der Walle 2008). They are further processed into variable dosage forms and used for sustained drug delivery at intracellular target sites (Bala et al.2004). Nano sizing (using liposomes as pharmaceutical nano-carriers) techniques improve the oral bioavailability of drugs with low aqueous solubility and permeability problems. These techniques also eliminate problems of drug resistance and allow free movement of drugs across barriers. Liposomes as nano sizing structures carry an additional advantage of being small in size with added flexibility and bio-compatibility (Fahr & Liu 2007).

### 1.3.2 Nanoparticles approach with poorly water soluble compounds – Advantages and Disadvantages:

Nano carriers in the form of liposomes, polymeric membranes are considered to be most efficient for the delivery of small and poorly water-soluble drug molecules through controlled release and localization of drugs at tissue and cellular cells (Bharadwaj et al.2005) (Delie, 1998). Molecular entities with solubility issues require technologies for enhancing drug solubility. Nano-carriers have potential to achieve desired formulations (wherein required quantities of the drug can be encapsulated) and are also useful for all routes of administration (Bala et al.2004). Nano sizing - a novel method of solubilisation improves bioavailability of drugs by delivering the contents at intracellular sites; however characterization of molecular targets, mechanism of targeted drug delivery still remains an issue (Bharadwaj et al.2005).

Manufacturing costs may be high when using nanoparticles which in turn could result in increase of formulation costs. Excessive use of polymeric molecules and toxic solvents may result in toxicity issues. The nanoparticle formulation of some low solubility drugs require addition of surfactants, other compounds (for better absorption) which can dilute the potency of drugs and can also result in enhanced cytotoxicity of cells.

## 1.4 Nanoparticle concept:

The field of nanotechnology dates back to 1980s, when microscopes originated which were capable of manipulating atoms and molecules on the nano-scale. Nanoparticles - the building blocks in nanotechnology are used to form different functional structures through microscopic control of particulate interfaces of various materials. Nanoparticles are sized between 1 and 100 nano-meters, exhibit properties that differ significantly from other materials and have potential applications in biomedical, pharmaceutical and other fields.

For over 20 years, nanoparticle concepts have been studied and their role in providing vast improvements in drug delivery techniques which minimize toxicity, improve efficacy and drug targeting have been acknowledged by researchers (Wim &Paul 2008).

Other concepts were explained which were related to drug delivery on crossing particular physical barriers and bioavailability at target intracellular sites. According to well-established concepts - drugs are either enclosed inside nanospheres or confined as nano-capsules where nanoparticles are covered by a polymeric membrane made of biocompatible and bio-degradable polymers. Biodegradable polymeric nanoparticles with novel properties and functions are considered as potential drug delivery systems due to their properties like controlled release, target ability to particular organ / tissue and ability to deliver required products after administration through per-oral route.

**1.5 Use of Nanotechnology to improve oral bioavailability of drugs:**

Oral route of drug administration, even though most preferred has few disadvantages as most drugs (usually protein/ peptide based) undergo degradation / rapid clearance at sites , activate an intestinal pump barrier ,are unable to cross the mucosal surfaces and also require accurate dosing-proportionality (Hite et al. 2003).

The oral bioavailability of a drug depends on several factors like solubility in aqueous medium (this being a major hurdle for majority of drugs), permeability, its rate of dissolution, pharmaco kinetic and pharmaco dynamic profile characteristics (Yellela, 2010). Potential drug delivery and targeting technologies like nanotechnology aim to minimize or prevent degradation, harmful side-effects, enhance bioavailability and controlled release at the site of action, especially through the per-oral route of drug administration. This technology can also be used to enhance the product characteristics of poorly soluble drugs through modulated formulation and can be easily incorporated into oral dosage forms also.

Nanotechnology can be used for enhancing the bioavailability of drug through per-oral means, without any chemical modification of the drug or altering normal physiology of the drug. Membranous cells in the follicle associated epithelium of the gastrointestinal tract are found to be the major site for drug uptake after absorption and translocation through systemic circulation. So, polymeric nanoparticles through oral route of administration can be targeted to these regions for increasing the bioavailability of the drug.

Nano-sizing/ Nanonization ,the technique in which, the particle size is reduced to the nano-meter range increases compound characteristics like saturation solubility and dissolution velocity for improved oral absorption of poorly soluble drugs. Nano - sizing is mainly used to improve the aqueous solubility of drugs (as saturation solubility, dissolution velocity increase resulting in improved absorption) thereby enhancing the oral bioavailability of poorly soluble API’s (Active Pharmaceutical Ingredients) or lipophilic drugs (Yellela, 2010).

### 1.6 Advantages of using a formulation of nanoparticles comprising a range of cardiovascular agents as a combination:

Cardiovascular agents are therapeutics used to control, regulate the complicated pathological processes involved in Coronary Artery Disease (CAD) like arrhythmias and other cardiac complications. Combination therapies or therapeutics belonging to the same class in combination are considered as effective avenues in treating all such complications when compared with high - dose therapy with a single drug.

Combination drug therapy (using beta- blockers and amiodarone) is especially found to be effective in treating frequent symptomatic episodes of cardiac events by providing maximum prevention with a significantly better survival rate. Such therapies offer treatment of life-threatening complications with fewer side-effects and also possess additive or even synergistic properties required for healing (‘Combination Pharmacotherapy for Cardiovascular disease’, 2005).

The possibility of administering combination drug cardiovascular therapies is an upcoming avenue for treating patients with atherosclerotic heart disease events, as the data reviewed from clinical studies confirms the efficacy and safety aspects (29–45% reduction in the risk of death was noted) of this mode of therapy. Combination of therapeutic agents or Combination Pharmacotherapy drugs (mostly preferred agents are statins, lipid / LDL absorption inhibitors, calcium-channel blockers, antihypertensive agents) which control Hypertension and LDL cholesterol greatly reduce the risk for major vascular events (‘Combination Pharmacotherapy for Cardiovascular disease’, 2005). These combination therapies are likely to be more effective with fewer side effects, with simplicity of administering a multiple-component pill and improved medication adherence (‘Combination Pharmacotherapy for Cardiovascular disease’, 2005).

A formulation of a plurality of nanoparticles comprising a range of cardiovascular agents as a combination (for administering a therapeutically effective amount of the formulation to a specific cardiac ailment) encapsulated within, adhered to a surface of nanoparticles are frequently used. A nanoparticle based approach is ideal for enhancing oral bioavailability or lowering the effective dose via formulating a range of cardiovascular agents into nanoparticles, as much lower doses of the active drug can be used, thus eliminating systemic toxicity.

## 2. Aim and objectives:

The first aim of this project was to develop isocratic HPLC technique for simultaneous estimation of furosemide and amlodipine drugs. They undergo through co-encapsulation and formulation into polymeric nanoparticles after they separated. Afterward, acquired nanoparticles will be characterized depending on their shape and size using zetasizer. This measures zeta potential of polymeric nanoparticles. That reason defines the goals for this study as performing in vitro drug release and using HPLC method to find drug entrapment efficiency.

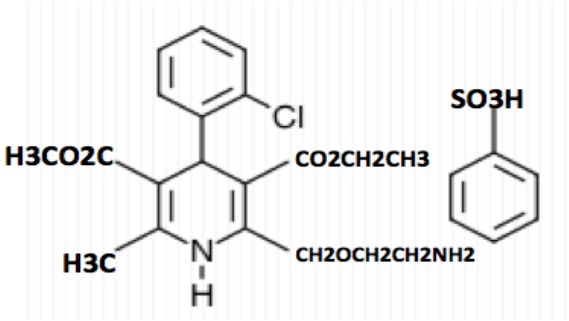
**3. Materials:**

**3.1 Drugs profile:**

**Amlodipine:**

This belongs to class II of BCS (high permeability and low solubility) (Merisko & Liversidge 2008), and is used to lower cardiac output or peripheral vascular resistance. It's a calcium channel blocker which hinders smooth muscle contractility and $Ca^{2}$+ entry with a relative absence of direct effects on the myocardium additionally it reduces myocardial oxygen and prevents coronary artery spasm. It's selectivity for calcium channel on vascular smooth muscle causes vasodilation ('Combination Pharmacotherapy for Cardiovascular disease', 2005). Amlodipine besylate was bought from Kemprotec Ltd. (UK). CAS 54-31-9, Batch No: F4381-5G and the chemical name is- 2-[(2- Aminoethoxy) methyl] -4- (2-chlorophenyl)-1, 4- dihydro-6-methyl-3,5pyridine dicarboxylic acid-3- ethyl 5- methyl ester. It has pka value 8.6, molecular weight: 567.1 and molecular formula: C20H25CLN2O5.C6H6O3S.

**Solubility**: in water (slightly) and in methanol (easily).




**Mechanism of action:**

Amlodipine lowers the arterial muscle contractility and consequent vasoconstriction through inhibition of calcium ions influx over L–type calcium channels. Calcium ions getting into the cell through these channels attach to calmodul. Calcium–bound calmodulin now attaches to and triggers the myosin light chain kinase (MILLIGRAMCK). There is a key step in muscle contraction where the triggered MILLIGRAMCK speeds up the phosphorylation of the governing light sequence component of myosin. Signals are amplified by calcium–induced calcium release from sarcoplasmic reticulum by ryanodine receptors. The contractile activity of arterial smooth muscle cells is lowered by the inhibition of the initial influx of calcium resulting in vasodilation. There is a decrease in blood pressure resulting from vasodilators effects of amlodipine. Mild can be treated to control exertion–related angina (chronic stable angina) and hypertension using amlodipine which is a long–acting CCB. It is

possible for carbonic anhydrase I action of vascular muscle to be affected by amlodipine making cellular pH rise. This might be used in controlling intracellular calcium inflow via calcium channel.

**Pharmacodynamics**:

It fits in dihydropyridine (DHP) group of calcium channel blockers (CCBs). This is the extensively used group of CCBs. In Homo sapiens, there are more than five category channels of calcium: N –, L –, P or Q –, T – and R – type. It is acknowledged that DHP CCBs focus on L–type calcium networks, which is the main passage in muscle cell facilitating contraction; nevertheless, certain studies have shown amlodipine binding and inhibiting N–type channel of calcium (see references in Targets section). Amlodipine attaches to dormant L–type calcium channels steadying their dormant conformation like other DHP CCBs. Inactive channels are extra prevalent in even muscle cells, as arterial even depolarization of the muscle is extensive in time than depolarization of cardiac muscle. Amlodipine extra arterial selectivity is obtained from alternate intertwining of the alpha– I sub unit of the channel.

At therapeutic sub–toxic concentrations, amlodipine has minimal outcome on cardiac myositis and transmission cells at therapeutic sub–toxic concentration.

**Pharmacokinetics**:

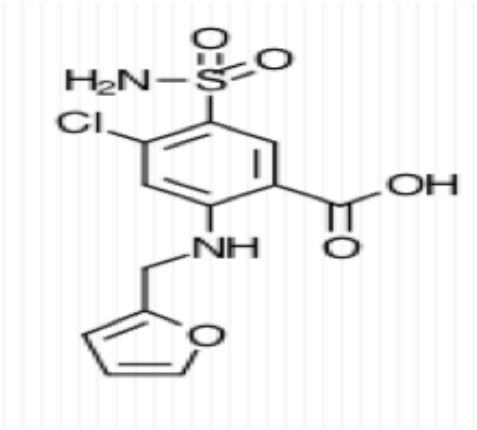
It is gradually and almost fully absorbed from the gastrointestinal region. Following an oral administration, maximum plasma concentrations are reached after 6 to12 hour. Bioavailability is estimated to be 64–90 percent. Food does not affect absorption. It metabolized widely (90 percent) to dormant metabolites through the cytochrome P450 3A4 ribozyme. 10 percent of the original composite and 60 percent of metabolites defecate in urine. Amlodipine has a half–life of 30 to 50 hours. Amlodipine is found to have 97.5 percent of protein binding.

**Furosemide:**

It fits into class IV of BCS (low permeability and high solubility) (Mohanchandran et al. 2010). It is used together with other groups of antihypertensive drugs. It is used to raise the urine flow. It is ring diuretic which hinders CL reabsorption in dense climbing loop of Henle. Additionally, it is used on patients suffering from congestive heart failure for their potency and rapid onset of action to lower pulmonary edema (‘Combination Pharmacotherapy for Cardiovascular disease’ , 2005). Furosemide was acquired from Sigma Aldrich. CAS 54-31-9, Batch No: F4381-5G and the chemical name is - 5-(aminosulfonyl)-4-

chloro-2-[(furanylmethyl) amino] benzoic acid. It has pka value 3.9,molecular weight: 330.7 and molecular formula: C12H11CLN2O5S.

**Solubility:** it is easily soluble in organic solvents like chloroform, ethanol, and methanol and basically in soluble in water.




**Mechanism of action**:

By hindering sodium–potassium–chloride cotransporter (NKCC2) in the dense climbing limb of Henle ring, furosemide, a ring diuretic, constrains reabsorption of water in nephron. This is attained via a good inhibition of the binding site of chloride in the cotransporter, thereby avoiding movement of sodium starting from the lumen of Henle ring into basolateral interstitium. Therefore, the intersection turns into low hypertonic while the lumen is extra hypertonic; this weakens water osmotic gradient of reabsorption via the nephron. Since the dense climbing limb is accountable for 25 percent reabsorption of sodium in nephron, furosemide is one of potent diuretics.

**Pharmacodynamics**:

It is a sulfonamide–type ring diuretic associated to bumetanide structurally. It is used to control hypertension as well as edema related to renal illness, cirrhosis, heart failure congestion, and nephrotic syndrome.

**Pharmacokinetics:**

60 percent absorbed in patients suffering from regular renal function. Merely, a little amount is metabolized hepatically to derivatives of defurfurylated, acid; 4–chloro–5–sulfamoylanthranilic. 95 percent furosemide destine into plasma proteins. Furosemide defecates in the urine. Considerably large furosemide defecates in the urine after I.V injection compared to after oral solution and after the tablet solution. Furosemide has a half–life of 2 hour.

**3.2 Polymer profile:**

Poly (lactic–co–glycolic acid) (PLGA, Resomer RG 50: 50 H; it has a molecular mass of 35–40KDa). It was acquired from – Boehringer Ingelheim (Ingelheim, Germany). It has long clinical practice thus the furthermost common amidst the different types of accessible polymers that are biodegradable. It has a good degradation features and likelihood for endured drug transport (Makadia et al, 2011).

Polyvinyl alcohol whose MW is 30 000 – 70 000 Da was used on PLGA Nano particles to stabilize for stability. It was bought from Sigma Aldrich (St. Louis, MO, USA). It has blending and cement behavior (Bala et al., 2005). A polyethylene glycol 400 is commonly used in various formulations of pharmaceutics like improving drug solubility (Bala et al., 2005) was bought from Fisher Scientific Limited (UK).

**3.3 Chemicals:**

Acetonitrile with CAS No: 75–05–8 has a molecular mass of 41.05 and (CH3CN) as its molecular formula. Ethyl acetate whose CAS No is 141–78–6 has a molecular mass of 88.11 and (CH3COOC2H5) as its molecular formula. Phosphoric acid whose CAS No is 7664–38–2. These chemicals came from Fisher Scientific Limited (UK).

## 4. Methods:

### 4.1 Simultaneous estimation by HPLC of class-II and class-IV drugs: Amlodipine Besylate and Furosemide:

#### 4.1.1 Preparation of solvents for mobile phase:

The solvent used for the mobile phase in the study was a mixture of phosphate buffer (pH3.0) and acetonitrile (ACN) in the ratio of 62:38. It was found to be the most suitable solvent for this study in separating Amlodipine and Furosemide (Nama et al., 2011).

A buffer containing phosphate was prepared by using 7 g of KH2PO4. The chemical powder was dissolved in 1000 mL HPLC grade water in a volumetric flask. It was then adjusted to pH3.0 by using phosphoric acid. To ensure smooth operation of the system, factors such as bubbles, insoluble must be removed. By using an ultrasonic water bath, the solvent mixture was degassed for 5 minutes and then under vacuum condition, it was filtered by using a membrane filter of 0.45 µm. Before finally applying to the column, the mixture was ensured completely homogenized by sonication (Nama et al., 2011).

#### 4.1.2 HPLC system conditions:

It's a chromatographic structure which was used. It has a great performance. Its product engineered by Thermo Separation, Hemel Hempstead.The experiment was carried out in room temperature. The prepared mobile phase solvent was pumped at 800 psi, resulting at 1 mL/min flow rate through the column, for no less than 30 min. When the mobile phase solvent has equilibrated the column, drug analytes were injected. Samples were processed for 6.5 min and drug detection was monitored at 220 nm (Nama et al., 2011).

**Table 1: Chromatographic conditions used after optimization**

| Parameter | Optimized Condition |
|---|---|
| Instrument | HPLC system |
| Column | ACE 5 C18-AR 5 µm, 150 mm×4.6 mm |
| Mobile phase | Phosphate buffer pH 3.0 : ACN (62:38) |
| Flow rate | 1 mL/min |
| Injection volume | 20 µl |
| Temperature | Ambient |

#### 4.1.3 Calibration plot:

Ten milligrams of Furosemide and 10 milligrams of Amlodipine besylate were liquefied in 2.5 milligram of mobile solvent phase (combination of phosphate buffer plus ACN, 40:60) in a volumetric container of 10 ml in volume. Additional 10 minute sonication was used to dissolve the drugs fully. A standard solution was made by filling the flask to 10 ml with solvent to 1000 µg/milligram concentration. Same steps were taken for Furosemide

and Amlodipine separately. Additional dilutions of 1 to20 µg/ml for furosemide and amlodipine were made using the solution in volumetric flasks of 10 ml with the diluent above. 20 µl of every concentration put three times in the column with 1 ml/min flow rate, and the equivalent chromatograms were acquired. The average area on the peak of every dilution was calculated from the chromatograms. The standardization graph made by marking drug concentration versus maximum area was established to be linear at the absorption array of 1 to 20 µg/ml. Calculations were done for the plot's regression equations. The equation was used to approximate the quantity of Furosemide and Amlodipine in the forms for tablet dosage (Nama et al., 2011).

**4.2 Preparation of Amlodipine and Furosemide PLGA nanoparticles:**

2.5 mL of ethyl acetate was used to dissolve 50 mg of PLGA and stirred for 2 hrs at 1000 rpm. 300 µl of drug-containing (2.5 mg of Amlodipine and/or 5 mg of Furosemide) PEG 400 was added to the dissolved PLGA. Then, the afore-prepared emulsification aqueous phase containing the PVA was added to each polymeric drug mixture. The mixtures were stirred (1000 rpm, 3 hrs) and homogenized (15000 rpm, 5 min) until the solution reached stable dispersion. The drug preparations were kept moderately stirred and 25 mL water was added to each. Same procedure was applied for preparation of amlodipine nanoparticles and furosemide nanoparticles. All samples were prepared in triplicate (Bala et al., 2005).

**4.2.1 Determination of size and electric charge of nanoparticles:**

In this study, the Malvern Zetasizer Nano (Malvern Instruments, UK) / Zetasizer 3000 (Malvern, UK) was used to determine the nanoparticles' size and zeta potential. With dynamic light scattering technique, the size was detected while zeta potential was measured under an electric field according to the principle of electrophoretic mobility. It was calculated by applying the Smoluchowski equation(Varshosaz & Soheili, 2008).

**4.2.2 Measurement of Drug Entrapment Efficiency:**

The amount of both drug entrapped in the nanoparticles were determined by the validated HPLC method. Briefly, drug loaded nanoparticles were centrifuged at 13000 rpm for 10 min and supernatant was diluted by using a mobile phase [Phosphate buffer (pH 3.0): ACN (62:38)].

20 µl of this diluted sample was injected to HPLC analysis (with the above mentioned optimized system conditions in Table 1) (Varshosaz & Soheili, 2008).

**4.2.3 In vitro drug release study:**

2.5 mg and 1.25mg furosemide and amlodipine drug nanoparticles were added in the 10 ml of phosphate buffer (pH 7.4) and stirred at 100 RPM. After 24 hour stirring the

nanoparticulate suspension was centrifuged at 13000 rpm for 15 min and the drug content was measured in supernatant as per the procedure in 4.2.2 (Derakhshandeh et al, 2020d).

## 5. Results:

### 5.1 Simultaneous estimation by HPLC of class-II and class-IV drugs: Amlodipine besylate and Furosemide:

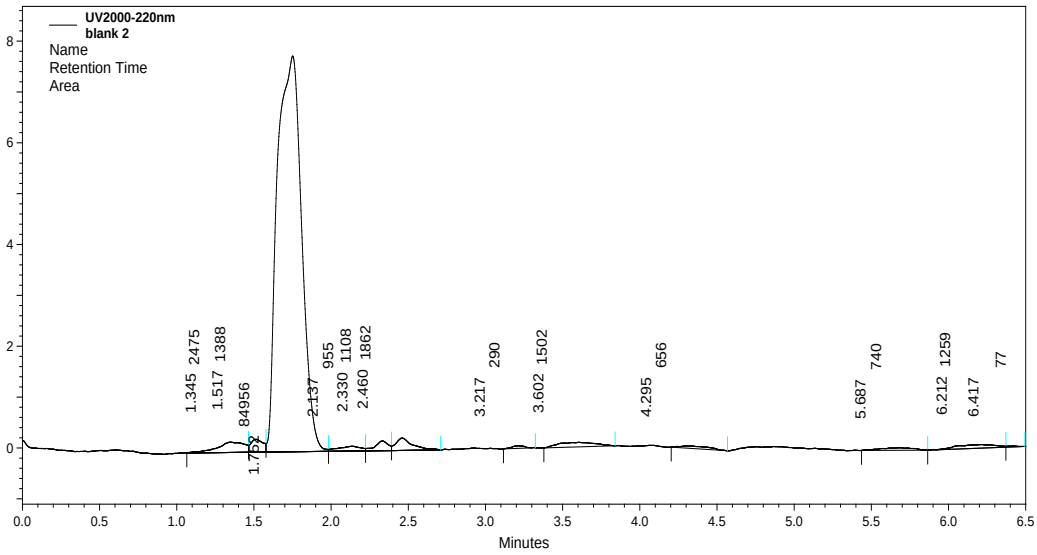



**Figure 1. HPLC chromatogram of blank sample (20 µL mobile phase)**

In the proposed isocratic HPLC method, ACE 5 C18-AR column was used with 1ml/min flow rate. Figure 1 showed typical HPLC chromatogram of blank sample (mobile phase 20 µL), and it was not shown any peak other than at 1.75 and it is considered as a solvent peak. These results indicated the absence of any contamination in the mobile phase.

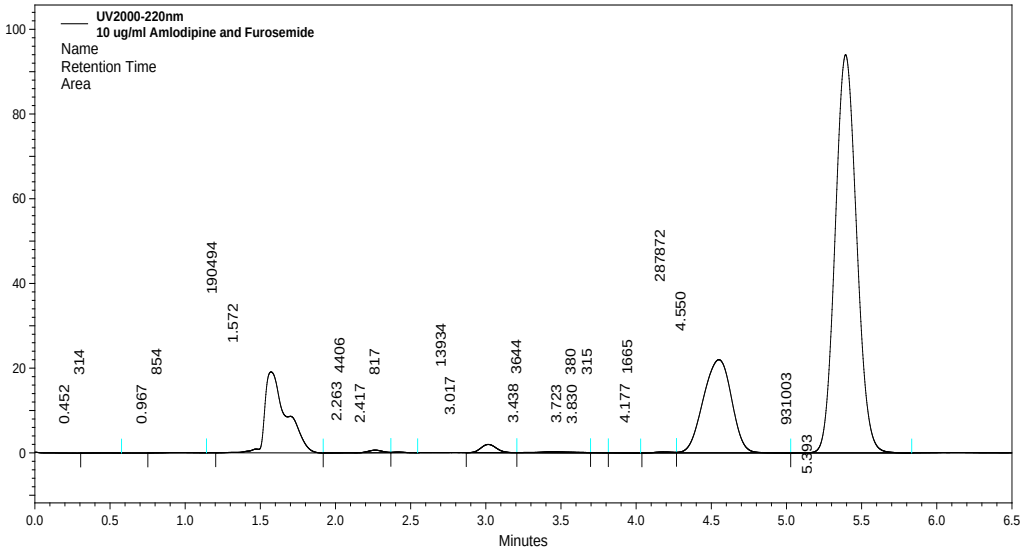



**Figure 2. HPLC chromatogram of Amlodipine and Furosemide (mixture)**

The mixture of Amlodipine and Furosamide showed two characteristic peaks at retention time of 4.55 min and 5.39 min (Figure 2).

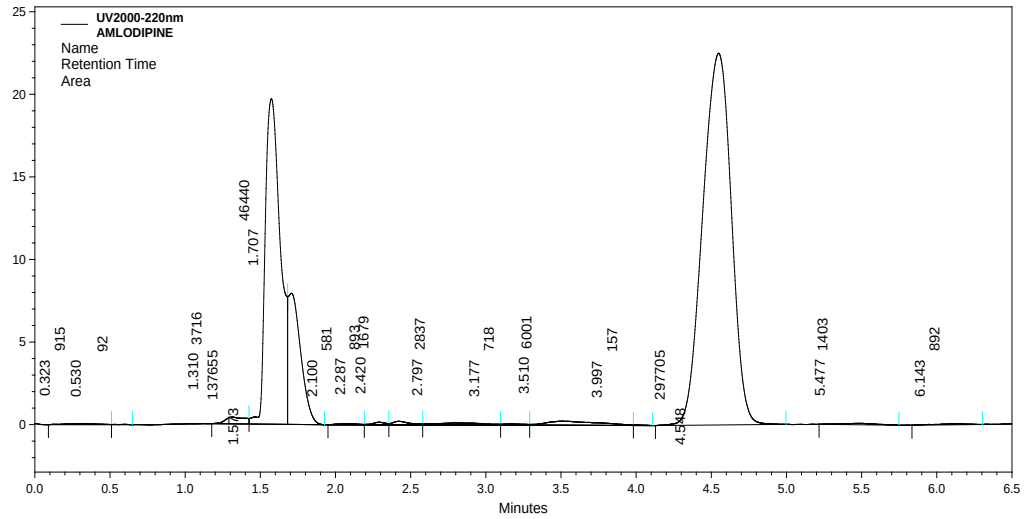



**Figure 3. HPLC chromatogram of Amlodipine**

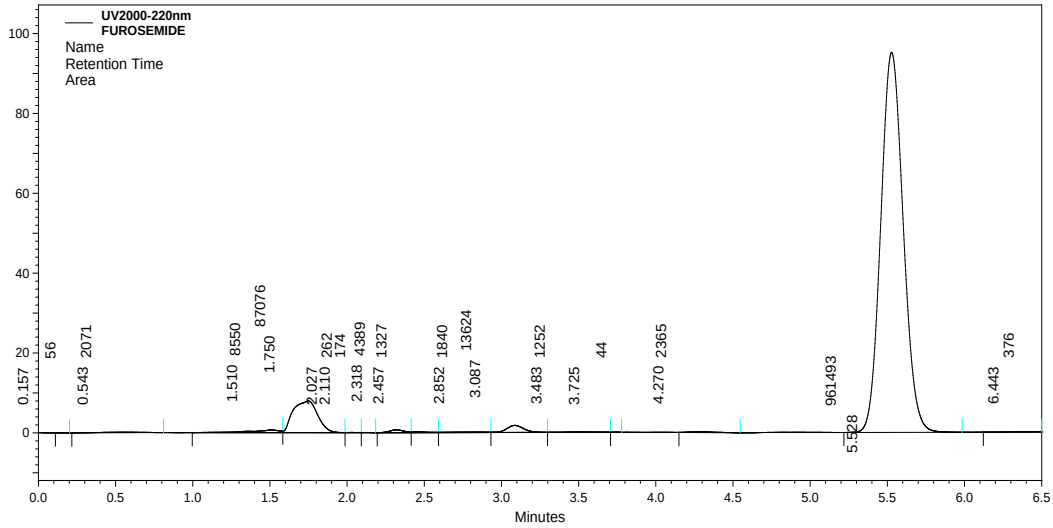



**Figure 4. HPLC chromatogram of Furosamide**

For validation of these two peaks, Amlodipine and Furosemide inserted independently to HPLC. It was validated that peak at retention time 4.54 min belongs to Amlodipine and peak at retention time 5.52 min belongs to Furosemide (Figure 3 and 4).

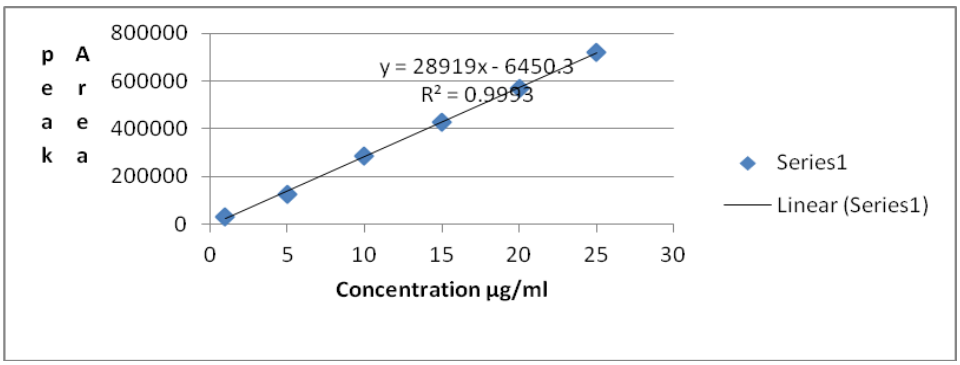



**Fig 5. Calibration plot of Amlodipine from mixture**

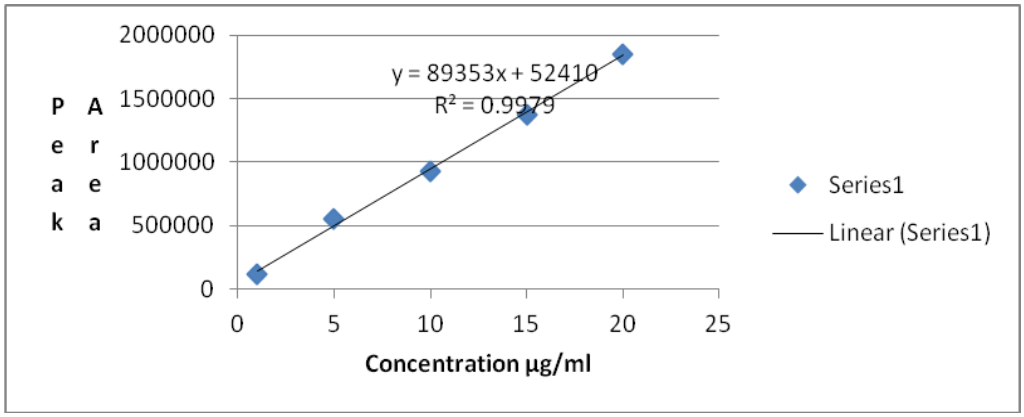



**Fig 6. Calibration plot of Furosemide from mixture**

For both Amlodipine and Furosemide, a linear range was obtained in the calibration plot (drug concentration against peak area). The range of concentration with the linear curve was1 – 20 µg/mL (Figure 5 and 6).

For concentration linearity plot, regression equation of Amlodipine and Furosamide above their peak areas were found to be Y=28919X+6450.3 ($R^2$=0.9993) for Amlodipine and Y=89353X+52410 ($R^2$=0.9979) for Furosamide , (with Y as the peak area and X the drug concentration µg/ml).

The optimized ration of acetonitrile: phosphate buffer (68:32 v/v) gave peak with decent shape and resolution. No disrupting peaks were established in the chromatogram of the mixture of both drugs. The proposed isocratic method is highly accurate as indicated by the high percentage of recovery. This isocratic HPLC method can be applied for many combination dosage forms of furosamide and amlodipine.

**5.2 Preparation of Amlodipine and Furosemide PLGA nanoparticles:**

**Table 2: Particle size, zeta potential and entrapment efficiency**

| **Formulation** | **Particle size (nm)** | **Polydispersity Index** | **Zeta potential (mV)** | **% Entrapment efficiency** |
|---|---|---|---|---|
| **Amlodipine Nanoparticles** | 234.0 ± 6.5 | 0.093 ± 0.008 | -11.8 ± 3.6 | 40 ± 10 |
| **Furosemide Nanoparticles** | 255.4 ± 4.3 | 0.098 ± 0.007 | -10.1 ± 2.1 | 58 ± 12 |
| **Amlodipine/furosemide Nanoparticles** | 236.1 ± 8.2 | 0.038 ± 0.002 | -7.12 ± 5.4 | 35 ± 6/49 ± 8 |

This table indicates several measurements for the three types of formulation mentioned above. These measurements are including the range of particle size in nm, polydipersity index, zeta potential and entrapment efficiency.

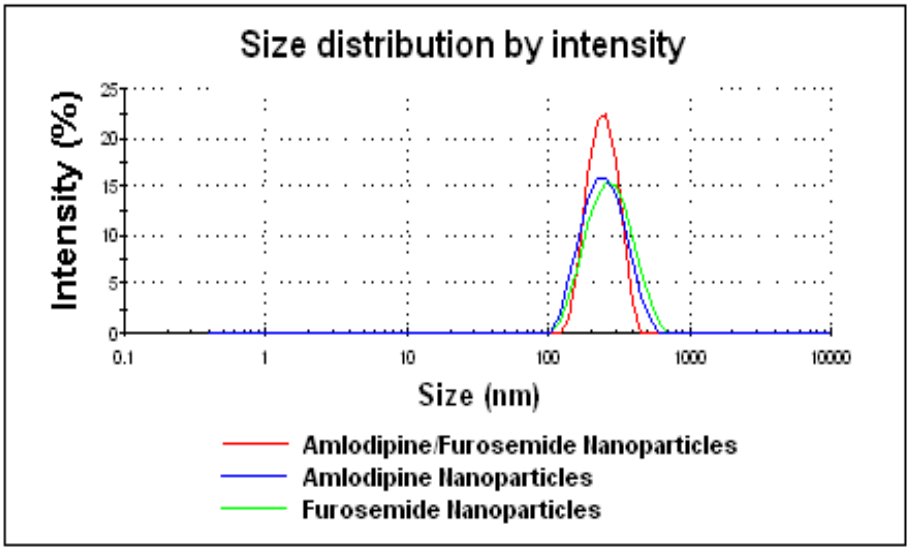



**Figure 7. Particle size distribution plot.**

This figure shows the size of each nanoparticle formulation. The Y-axis refers to the intenisty of light scattered from each formulation while the X-axis refers to their size in nanometer scale.

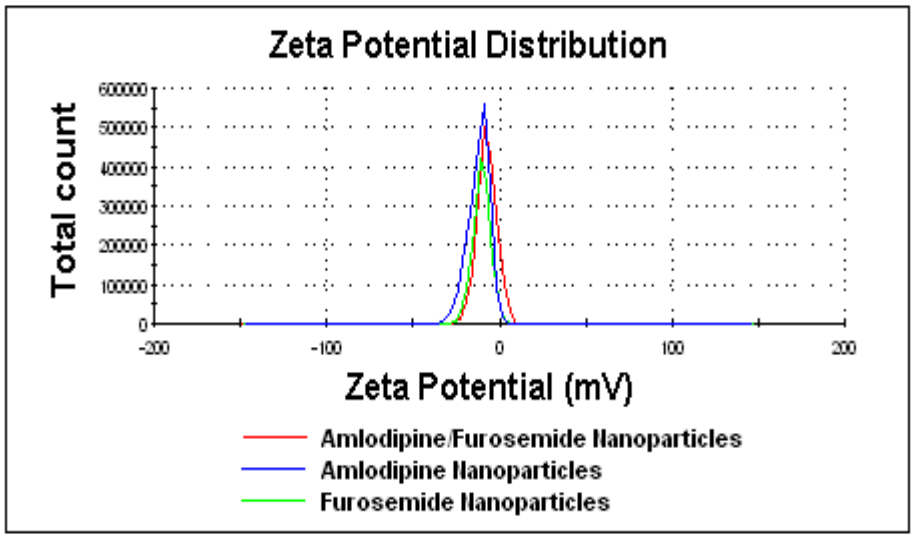



**Figure 8 .Zeta potential distribution**

This figure indicates the negative net charge of the three types of nanoparticles. Y-axis referes to the counts of molecules in each formation and the x-axis refers to the type of charges.

## 6. Discussion:

With the advances in the science field's particularly pharmaceutical one, different therapeutic drugs were manufactured. Unfortunately, there are problems associated with some therapeutics .For example cardiovascular agents that fall in the classes' I-IV (Bala et al.2004). These problems affect drug delivery system efficiency. Therefore, efforts were made to overcome BCS problems such as their low oral bioavailability and the need to the higher doses by formulating them into nanoparticles. For these reasons, several objectives were planned and mentioned in the introduction part of this project.

Originally, Isocratic HPLC method was proposed for the separation of Furosemide and Amlodipine from past preliminary studies (Nama et al , 2011). The blank chromatogram as in figure (1) revealed no peak, other than the peak at 1.75, which is a sign of the absence of any contamination in the mobile phase and separation of Amlodipine and Furosemide were effectively achieved at 4.54 min and 5.52 min respectively as in Figure (3) and (4).

Formulating furosemide and amlodipine in polymeric nanoparticles was the following stage. This was carried out to overwhelm problems such permeability rate limiting, gastric emptying rate limiting, both dissolution and permeability rate limiting, and dissolution rate limiting problems. This was performed by using polyethylene glycol 400 as a co-solvent because the Amlodipine was not soluble in ethyl acetate (Bala et al, 2005) and moved this drug containing PEG 400 in ethyl acetate in the presence of furosamide and PLGA. Additionally, polyvinyl alcohol (PVA) was used as a stabilizer for preparation of PLGA nanoparticles. PVA affect drug loading as well as release behaviour in addition to lowering the emulsion surface tension during preparation of nanoparticles (Bala et al, 2005). Moreover, amlodipine and furosemide nanoparticles were prepared through maintaining same composition.

Nanoparticles were then characterized depending to their shape and size using Zetasizer. It define the size using the Brownian movement (movement of molecules in a liquid) using the bombardment by the molecules by Dynamic Light Scattering (DLS). Size theory uses the speed of movement as a kind of measurement. Furthermore, Zeta potential theory measures particles movement in a liquid in an electrical field (Weiner et al., 1993; Fairhurst, 1994). Average particle size and zeta potential of furosemide nanoparticles, amlodipine nanoparticles and amlodipine/furosemide co-encapsulated PLGA nanoparticles were shown in table 2. Minor difference was observed in particle size of furosemide and amlodipine co-encapsulated nanoparticles as compared to furosemide and amlodipine nanoparticles (Figure 7) with lower polydispersity index measured the molecular mass in the

subsequent nanoparticle-drug mixtures and lower the value shows a well homogeneous dispersion, but any values larger than 0.3 proposes rising heterogeneity. Polyvinyl alcohol is the normally used stabilizer for the preparation of PLGA nanoparticles, which makes negative charge on the surface of nanoparticles ( Bala et al, 2005). Zeta potential is an important tool to measure the charge on the surface , and it has an essential involvement in the stability of suspension. For that reason, it has a major role in preparing a stable suspension.A colloidal molecules with low zeta potential value reveal a small charge that is needed for stabilisation and it should indicate marked stability vasus the added electrolytes. The negative zeta potential value for all the nanoparticles justifies the electrostatic stability of these nanoparticles as shown in Table 2 and Figure 8.

Drug entrapment of any drug is the ratio of the quantity of the drug entrapped into carrier system to the total quantity of the drug added (Ishihara, Goto, Kanazawa, Higaki, & Mizushima, 2009). Furosemide and amlodipine drug molecules are practically insoluble and soluble to a small percentage respectively. However, small entrapment efficiency was recorded in case of amlodipine as compared to furosemide (Table 2). No substantial variation was observed in between amlodipine/furosemide co-encapsulated nanoparticles and amlodipine and furosemide alone nanoparticles when loaded with 5% for Furosemide and 2.5% for Amlodipine.

The in vitro drug release study was tried once due to restriction of time given for this project. As a result, I could not reproduce additional results and confirm them. Different mechanisms involved in a drug release study; (i) surface and bulk degradation; (ii) disintegration; (iii) diffusion through the particle matrix; (iv) desorption of the surface-bound/adsorbed drug; (v) diffusion through the polymer wall, in case drug is encapsulated in the core; and (vi) a combined diffusion/degradation process (Derakhshandeh et al, 2020d). It was noted that co-encapsulated and alone encapsulated nanoparticle released the drug below 15% after 24 h incubation in phosphate buffer (pH 7.4) showed the drug release control.

## 7. Conclusion:

In conclusion from this study, the HPLC serves as an effective method for determining the quantity of Furosemide and Amlodipine. When compared to the other tested columns, the ACE 5 C18-AR column was found to be more suitable for the current experimental setup. Amlodipine and Furosemide were found from the used current preparations and conditions to have a retention time of 4.550 min and 5.393 min respectively. A linear range was observed from 1 to 20 µg/mL for both drugs. The regression equation was calculated (with Y as the peak area and x the drug concentration, µg/mL) from the calibration plot (concentration against peak area):

Amlodipine: $Y=28919\times + 6450.3$ ($r^2=0.9993$).

Furosemide: $Y=89353\times + 52410$ ($r^2=0.9979$).

Regarding the usage of PLGA nanoparticles in improving drug bioavailability, a plentiful quantity of effective drugs cannot be administered orally because of their low stability, membrane permeability, solubility, and unsuccessful efflux transport mechanisms. In view to this, new techniques are studied to promote the bioavailability of these drugs. One of these mechanisms makes use of the nanoparticle technology as established in the current study. Nanoparticles can be customised to have greater permeability and/or solubility. The encapsulation of class-II or class-IV drugs into these nanoparticles can improve their absorption into and metabolize by the body system. Problems with the drugs' original forms like as low aqueous solubility, enzymatic metabolism, permeation rate, efflux and first-pass effect can be worked out. This is definite in our findings that approximately 40% of Amlodipine and 58% of Furosemide were existed in the nanoparticles. The z-average particle size of Furosemide and Amlodipine co- encapsulated nanoparticles as compared to furosemide and amlodipine nanoparticles failed to show a large variation and the polydispersity was inside the acceptable range. For better bioavailability, these results propose that the BCS class-II and class-IV drugs can be encapsulated into nanoparticles.

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