Original Article

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Development and Validation of an *In Vitro* Dissolution Method Based on HPLC Analysis for L-Dopa Release From PLGA Nanoparticles

Optimize Edilen HPLC Yöntemi ile L-Dopa Yüklü PLGA Nanopartiküllerinden Etkin Madde Salımının Farklı *İn Vitro* Çıkış Yöntemleri ile Değerlendirilmesi

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ABSTRACT

Objective: In the past decade, dissolution testing has emerged as a valauble tool for the characterization of drug product performance in the field of pharmaceuticals. During the development of new formulations, dissolutions tests assist in the evaulation of any changes in the formulation arising during manufacturing process, thereby assuring product quality and performance post manufacturing.

Methods: In the present study, a simple high performance liquid chromatography (HPLC) method was developed and validated to quantitate the release of L-Dopa from poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles. The chromatographic separation was performed with a reversed-phase C18 column, using acetonitrile-water containing 0.05% trifluoroacetic acid (5:95, v/v) as a mobile phase at 280 nm. The developed method was validated for its specificty, linearity, accuracy, and precision according to the ICH guidelines.

Results: The developed method was shown to be linear (r2 ≥ 0.995) in the concentration range of 125-40 µg/mL. The mean % recoveries were found to be 102.59-98.70%, indicating an agreement between the true value and the detected value. Solution stability was guaranteed by the addition of an antioxidant. The analytical method was shown to be suitable for the evaluation of release of

ÖZ

Amaç: Farmasötik alanda çözünme hızı testleri, ilaç ürün performansını karakterize etmek için önemli parametrelerdendir. Üretim sürecinden sonra ürün kalitesini ve performansını sağlamak için, formülasyondaki değişiklikler, yeni formülasyonların geliştirilmesi sırasında çözünme testleriyle değerlendirilebilir.

Yöntemler: Bu çalışmada, poli (D, L-laktik-ko-glikolik asit) (PLGA) nanopartiküllerinden salınan L-Dopa miktarının belirlenmesi için yüksek basınçlı sıvı kromatografisi (HPLC) yöntemi geliştirilmiş ve onaylanmıştır. 280 nm hareketli faz olarak %0,05 trifloroasetik asit (5:95, h/h) içeren asetonitril-su kullanılarak, ters fazlı C18 kolonu ile kromatografik ayırma yapılmıştır. Geliştirilen yöntem, özgünlüğü, doğrusallığı, doğruluğu ve kesinliği için ICH kurallarına göre doğrulanmıştır.

Bulgular: Yöntemin 1,25-40 µg/mL konsantrasyon aralığında doğrusal (r2 ≥0,995) olduğu gösterilmiştir. Ortalama % geri kazanım, %102,59-%98,70 arasında olup, gerçek değer ile tespit edilen değer arasında bir korelasyon olduğu belirlenmiştir. Antioksidan ilavesiyle çözelti stabilitesi sağlanmıştır. Analitik yöntemin nanopartiküllerden salınan L-Dopa'nın değerlendirilmesi için uygun olduğu gösterilmiştir. Örnek ve ayırma (SS) ve diyaliz membranı (DM) yöntemleri kullanılarak *in vitro* salım çalışmaları

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L-Dopa from PLGA nanoparticles. *In vitro* release of L-Dopa was studied using sample and separate (SS) and dialysis membrane (DM) methods. To compare SS and DM methods, difference ($f1$) and similarity ($f2$) factors were calculated. No significant differences were recorded in the release kinetics of L-Dopa from nanoparticles using both methods ($f1$ <15 and $f2$ >50).

Conclusion: Dissolution test methods were compared and procedure for an analytical method based on HPLC was optimizated and validated for the dissolution of L-Dopa loaded nanoparticles.

Keywords: Analytical validation, L-Dopa, PLGA nanoparticles, *in vitro* drug release, sample and seperation method, dialysis membrane method

yapılmıştır. SS ve DM yöntemlerinin karşılaştırılmasında farklılık ($f1$) ve benzerlik ($f2$) faktörleri kullanılmıştır. Nanopartiküllerden L-Dopa'nın salım kinetiğindeki fark her iki yöntem için de anlamlı bulunmamıştır ($f1$ <15 ve $f2$ >50).

Sonuç: Bu çalışmada, L-Dopa yüklü nanopartiküllerden etkin madde salımının değerlendirilmesi için stabilite testleri de dahil olmak üzere bir çözünme testi yöntemleri karşılaştırılmış ve salınan L-Dopa miktarının tespiti için HPLC yöntemi optimize edilmiş ve valide edilmiştir.

Anahtar Sözcükler: Analitik validasyon, L-Dopa, PLGA nanopartikül, *in vitro* ilaç salımı, örnek ve ayırma yöntemi, diyaliz membran yöntemi

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder with a global prevalence of 1-3% in the population with age above 65 years. Clinical pathology of PD involves progressive loss of dopaminergic neurons, particularly in nigro-striatal area and its surrounding pathways. This loss of dopaminergic neurons affects movement and facial expressions in the affected patients (1,2). Conventional treatment for PD involves administration of L-Dopa, a dopamine precursor. However, L-Dopa therapy is associated with certain limitations. These include fluctuations in L-Dopa levels in the plasma owing to erratic gastric emptying and intermittent oral intake (3), poor relative bioavailability (~5-15%), and availability of <1% of the administered dose in the brain (4-7).

In the past few decades, several polymeric nanoparticles prepared using natural or synthetic polymers have been developed and explored to allow safe and enhanced delivery of drugs like L-Dopa to the targeted site. Among these, nanoparticles obtained from biodegradable and biocompatible polymers such as poly-lactic-co-glycolic acid (PLGA) or poly (glycolic acid) have been used most commonly. In general, nanoparticles are preferred for drug administration owing to their high chemical and biological resistance and ability to carry both hydrophilic and lipophilic substances in their active form. In addition, these nanoparticles can be administered in the body via different routes.

Since nanoparticles release small amounts of encapsulated drugs as a function of time, the analytical methods used to study *in vitro* release of drugs must be highly sensitive to quantify drug concentrations in the dissolution medium. Testing methods used to study *in vitro* release for nanoparticles based delivery systems can be broadly divided into three categories, membrane diffusion methods [such as dialysis method (DM)], sample and separation methods (SS), and continuous flow methods. Among these, DM is used most commonly to evaluate *in vitro* release of drugs from nanoparticles, followed by SS. In the present study, DM was used as reference method and SS was used as test method.

Among the currently available analytical methods, HPLC is the most commonly used method employed for the characterization of various pharmaceutical molecules. HPLC-diode array detector (DAD) offers a quick, sensitive, and accurate method for the separation and identification of drugs in pharmaceutical nanoparticulate formulations (8,9). Although several researchers have investigated *in vitro* release of drugs from nanoparticles, no HPLC method has been established for the simultaneous determination of L-Dopa. No studies have been reported for the use of DM and SS methods for the quantification of the amount of L-Dopa released from nanoparticles in dissolution medium. Besides this, in most of the reported studies used L-Dopa solutions at low pH as standard solution (10-13).

Nanoparticles were specially designed to ensure delivery of L-Dopa to brain by endocytosis when administered nasally. To represent dissolution profile of L-Dopa released from the nanoparticles, two buffered solutions mimicking pH conditions present in the endolysosomal compartment (pH 4.5) and brain (pH 7.4) were used (14). Several previous studies have reported evidences for the stability of L-Dopa in acidic conditions (10,12,13,15). The present study aimed to develop and optimize an analytical method to determine the levels of L-Dopa in different media having a higher pH as compared to the acidic media reported earlier for L-Dopa.

In the present study, a double emulsion-solvent evaporation method was used to prepare PLGA nanoparticles, wherein methylene chloride, polyvinyl alcohol, and PLGA were used as organic solvent, surfactant, and polymer, respectively. In order to determine the release profile of L-Dopa from PLGA nanoparticles, SS and DM methods were compared. In general, it is believed that *in vitro* release method reflects the changes in the manufacturing procedure that in turn affects the performance of the drug entrapped in the nanoparticles. Thus, *in vitro* dissolution test methods used in the study were compared and HPLC method was further validated to ensure the robustness of the developed analytical method. The study also included stability tests for the L-Dopa released from the PLGA nanoparticles.

Method

Instrumentation

For HPLC studies, Agilent 1100 series integrated HPLC system with DAD, pump, auto-sampler, and degasser unit was used. Reversed phase HPLC analysis was carried out using a 250x4.6 mm, 5 µm reversed-phase C18 HPLC column obtained from Macherey-Nagel. For *in vitro* drug release studies, orbital shaker and ultra centrifuge (Sigma® 30KS) purchased from Sigma were used.

Reagents and Materials

L-Dopa was a kind gift from ILKO Pharmaceuticals (Ankara, Turkey). Deionised water used in the study was obtained from a Millipore water supplier. Trifluoroacetic acid (TFA), NaCl, Na_2HPO_4, KH_2PO_4, acentonitrile (HPLC grade), and Tween 80 were procured from Merck. For HPLC analysis, the mobile phase was filtered through a 0.45 µm membrane filter (Millipore, Barcelona) and degassed using an ultrasonic bath, prior to use. Cellulose dialysis tubing (14,000 MWCO), polypropylene co-polymer centrifuge tubes, and polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich.

Chromatographic System and Conditions

First, the wavelength for absorption maxima for the drug was selected based on its UV spectrum. L-Dopa was characterized by a absroption maxima at 280 nm which was further used for HPLC analysis. Mobile phase for HPLC analysis comprised of TFA solution (0.05% v/v, pH 3) and acetonitrile at 95:5 (v/v). For chromatographic separation, 250x4.6 mm, 5 µm C18 reversed-phase HPLC column was used. A flow rate of 1 mL/min and run time of 7 minutes with 10 µL injection volume were used for HPLC analysis (1). The method used in the present study was developed and validated as per the considerations of the ICH guideline Q2 (R1) that involved several parameters including specificity, linearity, detection and quantification limits, repeatability and intermediate precision, accuracy, and stability (16).

Preparation of Reagents

Suitable analytical procedures should be used to define the amount of L-Dopa used during dissolution test. Two stock solutions of L-Dopa at concentration of 100 µg/mL were prepared by dissolving a suitable amount of L-Dopa in buffered solutions at pH 4.5 and pH 7.4. Prior to use, all solutions were sonicated for 30 min. For each stock solution, 6 diluted samples in the concentration range of 1.25-40 µg/mL (ppm) (1.25, 2.5, 5, 10, 20, and 40 µg/mL) were prepared from the stock solutions. The stability of L-Dopa in the diluents (phosphate buffer solution at pH 4.5 and pH 7.4) was investigated for a period of 48 hours at different tempratures (4 ºC, 25 ºC, and 37 ºC) in both the presence and absence of ascorbic acid.

Preparation of Nanoparticles

L-Dopa loaded nanoparticles were prepared using a double emulsion solvent evaporation method with PVA as a stabilizer. Briefly, L-Dopa and PLGA were first dissolved in dichloromethane (DCM). Further, distilled water was added to the resulting solution and mixed using an ultrasonic homogenizer to form a primary water-in-oil (W/O) emulsion. Following this, the primary emulsion was emulsified in PVA solution with homogenization to form a double water-in-oil-in-water (W_1/O/W_2) emulsion. The resulting double emulsion was stirred using a magnetic stirrer at a constant rate at room temperature (25 °C) to evaporate the organic solvent. The resulting nanoparticle suspension was further incubated at 4 °C overnight to ensure hardening of the PLGA matrix by allowing DCM to fully partition to the external aqueous phase. The nanoparticles were recovered by ultracentrifugation. The supernatant was carefully removed and pellet containing nanoparticles was washed twice with distilled water to remove free drug and excess surfactant. The sample was further subjected to lyophilization.

Dissolution Test Development

Dissolution Medium

The term "sink condition" is generally defined as the ability of the dissolution medium to dissolve at least three times the amount of drug present in the dosage form. Percentage of drugs released from the nanoparticles should be detected using the developed analytical method. Selection of the most suitable media conditions is based upon the stability of the analyte in the test medium and its application in terms of *in vivo* performance. Since the nanoparticles were specifically designed to deliver L-Dopa to brain via endocytosis using a nasal route of administration, the *in vitro* release of L-Dopa from PLGA nanoparticles was studied at pH 4.5 to mimic the endolysosomal pH and at pH 7.4 to mimic the brain pH (17). Phosphate buffer solution at pH 4.5 was prepared by dissolving 6.8 g potasium dihydrogen phosphate in 1 L distilled water containing 2 % Tween 80 (v/v) and 0.1 % (w/v) ascorbic acid (aa). Phosphate buffer solution at pH 7.4 was prepared by dissolving 2.38 g disodium hydrogen phosphate, 0.19 g potassium dihydrogen phosphate, 8 g sodium chloride, 2% Tween 80, and 0.1% aa in 1 L distilled water. In the present study, 2% (w/v) Tween 80 was used to enhance the solubility of L-Dopa in aqueous solution and 0.1% (w/v) aa was added to protect L-Dopa from oxidation during the assay (18).

Method devolopment

In Vitro Drug Release (SS Method)

L-Dopa loaded nanoparticles weighed to contain 45 µg L-Dopa were suspended in 5 mL of buffered solution. The nanoparticle suspensions were transferred to tubes and incubated in an orbital shaker bath at 37±0.5 °C with rotation at 100 rpm. The tubes were removed from the water bath at 30, 60, 120, and 240 minutes, and centrifuged at 2000 rpm for 20 min. The supernatant was carefully removed and used for further analyis. The nanoparticle pellets were resuspended in 5 mL of fresh buffer (pH 4.5) and placed back in the shaker bath. The supernatant was used to determine the concentration of L-Dopa. All experiments were perfomed in triplicates and results were expressed as mean ± variation.

In Vitro Drug Release (DM Method)

L-Dopa loaded nanoparticles containing 45 µg L-Dopa were suspended in 0.5 mL of buffered solution and inserted in a dialysis bag. The dialysis bag was placed in 4.5 mL (total 5 mL) of buffered solution at pH 4.5. This was further incubated at 37±0.5 °C in an orbital shaker bath at 100 rpm. To evaluate drug release as a function of time, 1 mL sample aliquots were collected at 30, 60, 120 and 240 minutes and replaced with 1 mL fresh buffer (pH 4.5). Drug concentrations in the aliquots were determined using the above mentioned analytical method. All experiments were performed in triplicates and results were expressed as mean ± variation.

Statistical Calculations

All results are reported as mean ± standard deviation of replicates. For drug release (%) at different time intervals, two tailed t-test was performed using Prism Software Version 6.0, where differences were considered to be significant with $p<0.05$. Microsoft Office Excel® was used to calculate $f1$ and $f2$ factors.

Results

The present study involved development of a HPLC based analytical method to determine time dependent release of L-Dopa from PLGA nanoparticles synthesized using a double emulsion solvent evaporation method. For reversed-phase HPLC analyis, mobile phase comprised of TFA solution (0.05% v/v) at pH 3 and acetonitrile at ratio of 95:5 (v/v). The chromatographic separation was carried out using a 250x4.6 mm, 5 µm C18 HPLC column. The flow rate for HPLC analysis was kept at 1 mL/min with run time of 7 minutes and 10 µL injection volume. No interfence was observed after injection of L-Dopa solution, placebo, and nanoparticles.

The regression equations for the calibration curve were found to be y=5.7424x+2.1421 (Figure 3) and y=7.4159x+9.5333 (Figure 4) for phosphate buffer solution at pH 4.5 and phospate buffer saline solution at pH 7.4, respectively. The regression coefficient r^2 was calculated to be 0.9998 and 0.995 for buffered solutions at pH 4.5 and pH 7.4, respectively. Linearity data calculated using Prism Software Version 6.0 are summarized in Table 1. Repeatability analysis for buffered solutions at pH 4.5 and pH 7.4 were characterized by relative standard deviation (RSD) % of 3.07-0.03% and 0.83-0.01%, respectively. For inter day assays, buffer at pH 4.5 was found to have precision with RSD % of 1.44-0.01% while buffer at pH 7.4 showed precision of RSD % 1.00% and 0.01%. The mean % recoveries for buffered solutions at pH 4.5 and pH 7.4 were calculated to be in the range of 102.59-98.70 and 101.92-100.00, respectively. L_D and L_Q values were found to be 0.0408 and 0.1235 µg/mL, respectively, for buffer at pH 4.5. For buffer at pH 7.4, L_D and L_Q values were 0.0636 and 0.1928 µg/mL, respectively. When the analysis was performed as function of temperature over a period of 48 hours, the amount of remaining L-Dopa was found to be 97.46% at 4 °C, 95.54% at 25 °C, and 89.63% at 37 °C for buffer at pH 4.5 in presence of ascorbic acid. In comparison to this, the remaining L-Dopa in buffered solution at pH 7.4 in the presence of aa over a period of 48 hours was calculated to be 103.21% at 4 °C, 88.21% at 25 °C, and 75.85% at 37 °C.

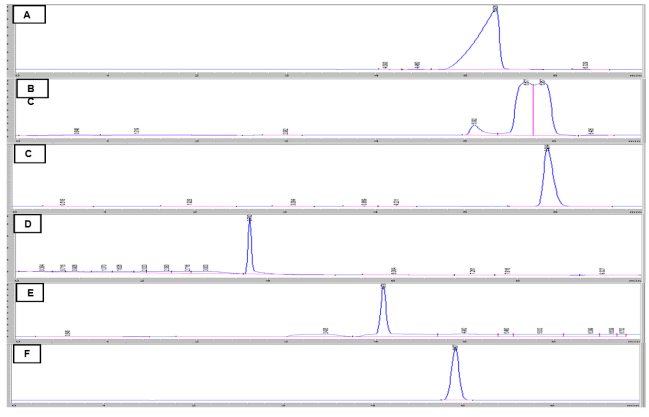
The differences in release rate were evaluated for SS and DM method. $f1$ was found to be 6.02 and $f2$ was 95.38. No differences were observed in the test and reference methods for all the time intervals for which drug release (%) was studied ($p=0.853$; $p<0.05$).

Discussion

Optimization of the Chromatographic Method

The HPLC method developed in the present study to determine the release of drugs provided a reliable quality control analysis. The wavelength selection for HPLC analysis was done on the basis of absorption maxima obtained for three different concentrations of L-Dopa solutions according to the acquired ultraviole (UV) spectra. A wavelength of 280 nm was selected for HPLC analysis because it provided high sensitivity, required for the quantitation of significantly low concentations of the drug present in the dissolution samples.

The detection of L-Dopa required an adequate mobile phase comprising of a suitable ratio of polar to non-polar solvent. For an acceptable chromatographic separation, several parameters including pH of the mobile phase and percentage of organic modifier were tested. HPLC analysis was conducted at a flow rate of 1 mL/min with a run time of 7 minutes and 10 µL injection volume. L-Dopa at a concentration of 10 ppm was injected into the system. The chromatograms obtained for the HPLC analysis showed that the use of acidic mobile phase with reversed-phase C18 column provided high solubility for L-Dopa resulting in



	Chromotographic conditions	Retention (time minute)	Symetry of peak	pH of TFA
A	T FA - acetonitrile (95 - 5)(v/v)	5.3	5.514	7.6
B	TFA - acetonitrile (90 - 10)(v/v)	-	-	7.6
C	TFA - acetonitrile (95 - 5)(v/v)	5.9	0.666	2,3
D	TF A - acetonitrile (90 - 10)(v/v)	4.1	0.61	2,3
E	TFA - acetonitrile (97 - 3)(v/v)	3.7	0.784	2,3
F	TFA - acetonitrile (95 - 5)(v/v)	4.9	1.058	3

Figure 1. Preliminary chromatogram for HPLC analysis performed on a C18 reverse phase column

HPLC: High performance liquid chromatography

symmetric and sharp peaks. As shown in Figure 1, TFA solution was determined as acidic buffer solution for HPLC analysis.

For the mobile phase used in HPLC analysis, when the ratio of acetonitrile to 0.05 % (v/v) TFA solution in water was changed from 10:90 (v/v) to 5:95 (v/v) a sharp peak for the active substance was obtained. Following this, a ratio of 2.5:97.5 (v/v) for acetonitrile to 0.05% (v/v) TFA solution was also tested for mobile phase to allow for better separation. However, peak of L-Dopa couldn't be determined at this ratio in contrast to our previous experiments. In order to protect the column's integrity, pH for 0.05% (v/v) TFA solution was adjusted to pH 3 using 0.1 N HCl solution and thus the resulting peak tailing was acceptable (Figure 1).

For the sample peak, symmetry factor was found to be 1.058 which was within the limit of <1.5 as established for various pharmacopoeias (Figure 1). Therefore, all the HPLC analysis performed in the study were carried out using a 250x4.6 mm, 5 µm C18 HPLC column with a flow rate of 1 mL/min, run time of 7 minutes, 10 µL injection volume, 25 °C column temperature, and mobile phase comprising of acetonitrile and 0.05% (v/v) TFA solution at 5:95 (v/v) (pH 3) (1).

Nanoparticles used in the study were specifically designed to be transported to brain via nasal route by means of endocytosis. To evaluate the dissolution profile of L-Dopa from PLGA nanoparticles, two buffered solutions at pH 4.5 and pH 7.4 were used to mimic pH of endolysosomal compartment and brain, respectively (14). As mentioned earlier, several studies have previously established the stability of L-Dopa in acidic conditions (10,12,13,15). Therefore, present study focussed on developing and optimizing a HPLC based analytical method to determine the amount of released L-Dopa in different media conditions having pH values higher than those reported in previous studies. In addition to this, buffered solutions at pH 4.5 and pH 7.4 with and without L-Dopa were also injected and analyzed using the same method to check for any interference in the peaks. As shown in Figure 2, no interference was observed (Figure 2).

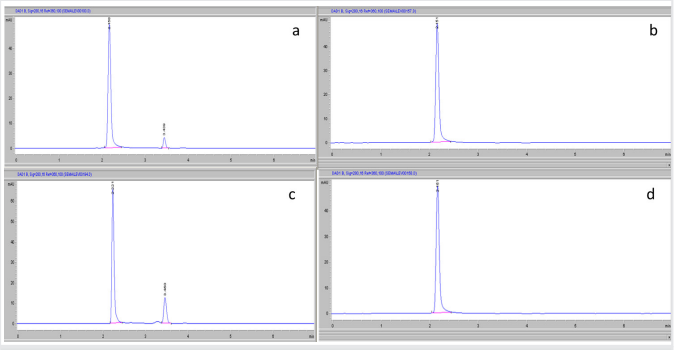


Figure 2. a) Chromatogram of L-Dopa in buffered solution at pH 4.5, b) Chromatogram of buffered solution at pH 4.5, c) Chromatogram of L-Dopa in buffered solution at pH 7.4, and d) Chromatogram of buffered solution at pH 7.4

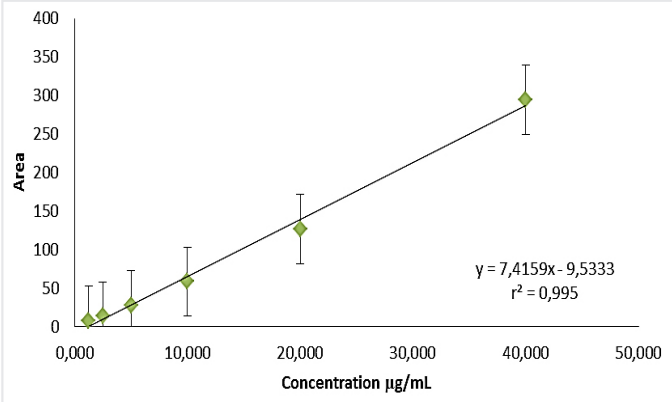


Figure 4. Calibration curve of L-Dopa in buffered solution at pH 7.4 obtained using HPLC based analytical method (n=3)

HPLC: High performance liquid chromatography

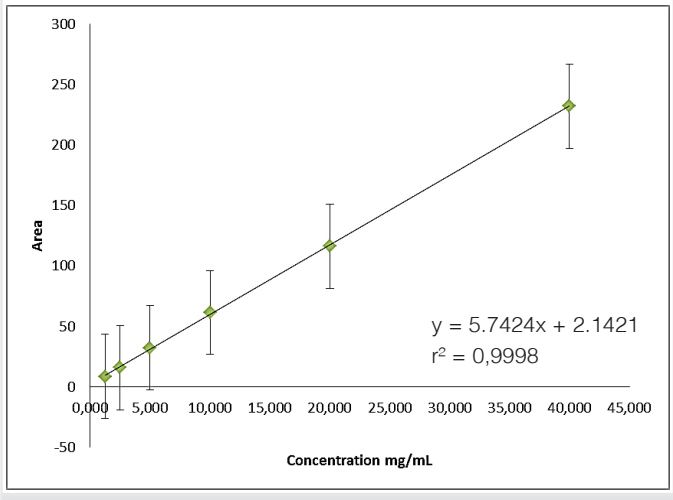


Figure 3. Calibration curve of L-Dopa in buffered solution at pH 4.5 obtained using HPLC method (n=3)

HPLC: High performance liquid chromatography

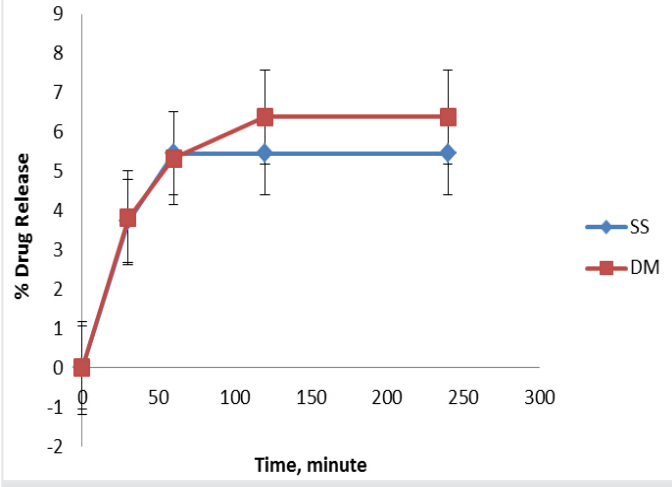


Figure 5. Comparative release profile of L-Dopa from nanoparticles obtained using DM and SS methods (n=3)

DM: Dialysis method, SS: Separation methods

Method Validation

In the present study, the analytical method was developed and validated according to the ICH guideline Q2 (R1) (16). The developed method was validated for various characteristics including linearity, accuracy, precision, specificity, stability, and detection limit and quantification limit. The method thus established was further utilised to quantitate the amount of the drug released from L-Dopa loaded nanoparticles.

Linearity

For the evaluation of linearity, standard solutions at different concentrations of L-Dopa were used. ICH guidelines suggest use of a minimum of five concentration levels for linearity studies. In the present study, linearity for L-Dopa was determined over a range of 1.25-40 µg/mL at six different concentrations. All the analysis were performed in triplicates (n=3) (16). For each concentration, samples were analysed six times, and the resulting peak areas were documented and analysed. The results for the regression analysis are shown in Figures 3 and 4. A good linear relationship ($r^2 \geq 0.995$) was observed between the concentrations and their respective peak areas as provided by the detector (Figures 3 and 4). The regression coefficient r^2 values ≥0.995 are considered to be acceptable according to the ICH guidelines (16). Generally, correlation intercept is calculated to evaluate the acceptability of the linearity of the data. In fact, for better analysis of the data, slope of line should also be calculated (Table 1) (8). All these results showed that all calibration curves were characterized by suitable linearity according to ICH guidlines (16).

Precision

The values for the absolute differences between the mean assay results for the developed method were obtained using repeatability and intermediate precision tests. All these values met the acceptance criteria of RSD %, RSD <2.0. In general, repeatability establishes the precision of multiple sampling under the same operating conditions over a short interval of time. In comparison to this, precision establishes variations arising from the same laboratory under variable operating conditions, for example different days, analysts or equipment. Thus, all these results suggest that the developed method was reproducible and precise (19).

Repeatability

Repeatability proves precision of the method under same operational conditions over a short period of time. Repeatability also refers to intra-assay sensitivity. In the present study, low (1.25 µg/mL), medium (5 µg/mL), and high (20 µg/mL) concentrations of L-Dopa used in the calibration curve were analyzed to determine mean, standard deviation (SD), and RSD (n = 6). As per the standard guidelines, RSD % for standard peak must be < 2.0 (20). As shown in Tables 2 and 3, the developed method met the standart requirements of this analytical validation parameter for medium (5 µg/mL) and high (20 µg/mL) concentrations of the drug (20). In case of L-Dopa concentration of 1.25 µg/mL, which represents the lower concentration, % RSD was >2.0. However, there are several studies that extend the limits for % RSD to 5-6% (8). Since the concentration of 1.25 µg/mL represents the lowest point of the calibration curve, so extension of limits might be suitable for this method.

Intermediate Precision

The variations in terms of analyst for the developed method were determined using replicate injections of the above mentioned concentrations and analyzed using different analysts on the same day (Tables 4 and 5). Intermediate precision was performed using

Table 1. Linearty data for analytical method

Values	pH 4.5	pH 7.4
Slope	5.742	7.416
Standard deviation of slope	0.02059	0.1312
Confidence interval 95%	5.699-5.786	4.679-0.896
Correlation coefficient	0.9999	0.9975
Regression coefficient	0.9998	0.9950
Intercept	2.142	-9.533
Standard deviation of Intercept	0.3595	2.473
Confidence interval of intercept	1.390-2.895	-14.777- -4.290

Table 2. Repeatability analysis for the assay performed in buffered solution at pH 4.5 (n=6)

1.25 µg/mL		5 µg/mL		20 µg/mL	
Area	**Concentration µg/mL**	**Area**	**Concentration µg/mL**	**Area**	**Concentration µg/mL**
Average	1.24863	**Average**	5.12961	**Average**	19.73933
SD	0.04	SD	0.03	SD	0.13
RSD	3.07	RSD	0.50	RSD	0.68

SD: Standard deviation, RSD: Relative standard deviation

RSD % of six repeated assays on samples at three concentration levels. RSD % values were found to be in the range of 1.44-0.1% for all concentration levels and pHs studied. For intermediate precision, RSD % value should not exceed 2.0% The results summarized in Tables 4 and 5 show that the developed method met the requirements set for this analytical validation parameter (16).

Accuracy

The accuracy was determined in terms of recovery of known amounts of L-Dopa. For testing the accuracy of analytical methods, three concentrations (1.25, 5, and 20 µg/mL representing low, medium, and high concentrations, respectively) of the drug, covering the linear range of analytes, were prepared by diluting the stock solutions. The mean % recoveries were found to be in the range of 101.92-98.70%, thus implying an agreement between the true value and the found value (Tables 6 and 7). All these results indicate that the developed method met the requirements for method verification according to ICH guidelines (16).

Specificity

Specificity of an analytical method can be defined as the ability to assess an analyte unequivocally in the presence of expected components. This definition has a descriptive effect on the identity of an analyte. In our study, the specificity tests were performed using three different polymers for nanoparticle preparation and L-Dopa in two different media conditions. It was decided that the HPLC method was specific for the determination of L-Dopa according to the data summarized in Table 8. L-Dopa samples were injected five times and similar retention times were observed in all cases. All the nanoparticles prepared using different polymers were injected with or without L-Dopa. No interference was observed between the peaks of nanoparticles and L-Dopa. Thus, the described HPLC method was specific for buffered solution at pH 7.4 buffer and selective for buffered solution at pH 4.5.

Table 3. Repeatability analysis for the assay performed using HPLC based analytical method with buffered solution at pH 7.4 (n=6)

1.25 µg/mL		5 µg/mL		20 µg/mL	
Area	**Concentration µg/mL**	**Area**	**Concentration µg/mL**	**Area**	**Concentration µg/mL**
Average	1.31923	**Average**	5.00051	**Average**	18.49404
SD	0.01	**SD**	0.04	**SD**	0.10
RSD	0.56	**RSD**	0.83	**RSD**	0.53

SD: Standard deviation, RSD: Relative standard deviation, HPLC: High performance liquid chromatography

Table 4. Precision analysis of the assay performed using HPLC based analytical method with buffered soltuion at pH 4.5 (n=6)

	1.25 µg/mL		5 µg/mL		20 µg/mL	
	1. day	**2. day**	**1. day**	**2. day**	**1. day**	**2. day**
Average	1.2128	1.19172	5.12961	4.89316	20.6562	20.6382
SD	0.01	0.02	0.03	0.05	0.77	0.02
RSD	1.44	1.58	0.5	1	0.66	0.1

SD: Standard deviation, RSD: Relative standard deviation, HPLC: High performance liquid chromatography

Table 5. Precision analysis for the assay performed using HPLC based analytical method with buffered soltuion pH 7.4 (n=6)

	1.25 µg/mL		5 µg/mL		20 µg/mL	
	1. day	**2. day**	**1. day**	**2. day**	**1. day**	**2. day**
Average	1.22944	1.17172	5.00051	4.8931600	19.49404	19.63820
SD	0.01	0.02	0.04	0.05	0.10	0.02
RSD	0.54	0.85	0.83	1.00	0.53	0.11

SD: Standard deviation, RSD: Relative standard deviation, HPLC: High performance liquid chromatography

Table 6. Accuracy analysis for the assay performed using HPLC based analytical method with buffered soltuion at pH 4.5 (n=6)

1.25 µg/mL		5 µg/mL		20 µg/mL	
Experimental concentration	**% Recovery**	**Experimental concentration**	**% Recovery**	**Experimental concentration**	**% Recovery**
Average	99.89	**Average**	102.59	**Average**	98.70
SD	1.06	**SD**	0.51	**SD**	0.67
RSD	1.07	**RSD**	0.50	**RSD**	0.68

SD: Standard deviation, RSD: Relative standard deviation, HPLC: High performance liquid chromatography

Stability

The stability of L-Dopa in aqueous solution was evaluated to verify any spontaneous degradation of the samples during preparation (21). The aqueous solutions of L-Dopa were found to be unstable. However, aqueous solutions have been previously shown to be stable in the presence of high concentrations of acidic substances. Stability of L-Dopa remained uneffected in the presence of light. The storage condition for the active substance was specified to be at 2-8 °C. In addition to this, the oxidation of L-Dopa can be avoided in the presence of antioxidants. Therefore, information was obtained for the stability of L-Dopa in the presence of aa in the body and during the validation and formulation studies (3,18,22). Stock solution of L-Dopa at 0.4 mg/mL concentration was prepared using buffered solutions at pH 4.5 and pH 7.4. The solutions were mixed and vortexed until solid particles disappeared. The samples were further divided into aliquots of 3 mL. The stability of the solutions containing ascorbic acid was extended up to 48 hours. No oxidation was observed in the solutions containing aa at 4 °C. Thus, all these results suggest that aa should be added as a preservative in the dissolution medium during dissolution studies. No interference was recorded between the peaks of L-Dopa and ascorbic acid. The results of stability studies are summarized in Tables 9 and 10.

Table 7. Accuracy analysis for the assay performed using HPLC based analytical method with buffered soltuion pH 7.4 (n=6)

1.25 µg/mL		5 µg/mL		20 µg/mL	
Experimental concentration	**% Recovery**	**Experimental concentration**	**% Recovery**	**Experimental concentration**	**% Recovery**
Average	101.92	**Average**	100.00	**Average**	100.01
SD	0.74	**SD**	0.81	**SD**	0.83
RSD	0.56	**RSD**	0.81	**RSD**	0.83

SD: Standard deviation, RSD: Relative standard deviation, HPLC: High performance liquid chromatography

Table 8. The results of specificity analysis for HPLC method developed for L-Dopa

	pH 4.5		pH 7.4	
	Time (minute)	**Area**	**Time (minute)**	**Area**
L-Dopa	4.2	42.7	3.93	39.9
Resomer RG 503 H nanoparticles	2.3	10	0	0
Resomer RG 756H nanoparticles	2.3	8.6	0	0
Resomer RG 756H nanoparticles	2.4	9.4	0	0

HPLC: High performance liquid chromatography

Tablo 9. Results for stability study of L-Dopa in buffered solution at pH 4.5 (aa = ascorbic acid)

	pH 4.5					
	aa added			aa not added		
Hours	**0**	**24**	**48**	**0**	**24**	**48**
Temperature	**% drug content**			**% drug content**		
4 °C	100.00	100.01	97.46	100.00	100.17	68.37
25 °C	100	99.27	95.54	100.00	100.02	60.64
37 °C	100	100.29	89.63	100.00	100.05	55.89

Tablo 10. Results for stability study of L-Dopa in buffered solution at pH 7.4 (aa = ascorbic acid)

	pH 7.4					
	aa added			aa not added		
Hours	**0**	**24**	**48**	**0**	**24**	**48**
Temperature	**% drug content**			**% drug content**		
4 °C	100.00	100.07	103.21	100.00	98.55	87.96
25 °C	100.00	104.20	88.21	100.00	86.47	70.70
37 °C	100.00	99.96	75.85	100.00	88.78	67.95

Limit of Detection (L_D) and Limit of Quantification (L_Q)

Limit of detection (L_D) represents the lowest concentration level in a peak area where signal level is atleast three fold of the baseline-to-noise. Limit of quantification (L_Q) represents the lowest concentration level that can be precisely provided by a peak area with a given signal-to-noise. Calibration curve was calculated using L-Dopa concentrations in the range of 1.25-40 μg/mL. The following equations were used:

Detection limit (L_D) =3.3 a/s;

Quantification limit (L_Q) =10 a/s;

where "a" is the standard deviation of y-intercept of regression lines and "s" is the slope of the calibration curve. For L_D and L_Q, no defined limit is available in the literature and these are specific for each method. As shown in Table 12, the results met the requirements for *in vitro* dissolution tests (23).

***In Vitro* Drug Release**

In order to design a suitable dissolution medium for a poorly soluble drug, the first method involves increasing the volume of aqueous sink conditions or decreasing the amount of dissolved drug. The second approach is based upon the addition of anionic or non-ionic surfactans and solubilization of the drug by co-solvents up to 40%. In another approach, pH is altered to obtain a better sink condition. The last two approaches are less cumbersome and have been employed more widely in dissolution tests in the pharmaceutical industry. To enhance the solubility of L-Dopa 2% Tween 80 was added to the dissolution medium. In addition to this, ascorbic acid was also used in the mediums to protect L-Dopa against oxidation during *in vitro* release.

The cumulative amount of L-Dopa released from polymeric material was plotted as a function of time. As shown in Figure 4, only 5-6% of the drug content was released from the nanoparticles as evaluated for both methods. In order to achieve sink conditions, L-Dopa concentrations shouldn't exceed 20% of its saturation solubility in dissolution medium which was maintained in the the present study. Thus, inhibited release of L-Dopa was not contributed by insufficient sink conditions. In addition, it did not arise owing to inadequate loading of nanoparticles either (amount of released drug as a function of the encapsulated drug substance). Interactions between L-Dopa and PLGA prevented complete dissolution. Moreover, 5-6% of the drug content was suitable enough for the dicussion of the two methods. *In vitro* release profiles of the test formulations were similar to the reference formulation. In literature, it is mentioned that release rate of the encapsulated drug is progressively higher in SS method as compared to DM (Figure 5) (24). This might be contributed by the differences in the hydrodynamics of the system as the nanoparticles are present in the dialysis bag in one methode while in the other formulation is dispersed in a flask (25).

The similarity factor ($f2$) is a measurement of the similarity in the percent (%) dissolution between the two profiles (26). The difference factor ($f1$) is proportional to the average difference between the two profiles. FDA guidelines recommend that $f1<15$ and $f2>50$ are indicative of equivalence in dissolution profiles (27).

$$f2 = 50 \times \log [1 + (1/n) \Sigma^{n}_{t=1} (R_t - T_t)^2]^{-0.5} \times 100)$$

$$f1 = [\Sigma^{n}_{t=1} (R_t - T_t) / \Sigma^{n}_{t=1} R_t] \times 100$$

As shown in Table 12, no signicant differences were obtained in the release kinetics of L-Dopa as studied using both methods ($f1<15$ and $f2>50$) (24,28). A two tailed t-test was used to compare the dissolution profiles. No differences were observed in the drug release (%) for the test and reference methods at all time intervals ($p=0.853$; $p<0.05$).

Table 11. Limit of detection (L_D) and Limit of quantification (L_Q) for HPLC analysis

	L_D	L_Q
pH 4.5	0.0408	0.1235
pH 7.4	0.0636	0.1928

HPLC: High performance liquid chromatography

Conclusion

The analytical methods developed and validated in the present study were found to be simple, sensitive, accurate, and precise. The results of the study indicated that the developed methods are suitable for dissolution studies as well as routine quality control analysis of L-Dopa present in nanoparticulate formulations. DM and SS methods were characterized by similar *in vitro* release

Table 12. Values for f_1 and f_2 factors for the dissolution profile obtained using DM as reference and the SS as test model (n=3)

	SS method	DM method
Time (minute)	Test model, drug release (%)	Reference model, drug release (%)
30	3.73±0.03	3.81±0.01
60	5.45±0.02	5.33±0.05
120	5.45±0.02	6.38±0.01
240	5.45±0.02	6.38±0.01
	f_1	**6.02**
	f_2	**95.38**

DM: Dialysis method, SS: Separation methods

profiles, with acceptable precision (<10% SD). SS method showed faster release from formulations as compared to those observed using dialysis bag (Table 12). No significant differences were reported between DM and SS methods used for release kinetics study ($f1<15$ and $f2>50$). For further studies, the optimal method with *in vivo* relevance needs to established keeping into consideration *in vivo* to *in vitro* corelation.

Ethics

Ethics Committee Approval: *In vitro* study.

Informed Consent: *In vitro* study.

Peer-review: Internally and externally peer reviewed.

Authorship Contributions

Data Collection or Processing: S.A., Ö.S., Analysis or Interpretation: T.Ç., Literature Search: S.A., Ö.S., Writing: S.A., T.Ç.

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