Evaluation Of Release Of L-DOPA From PLGA Nanoparticles With Different In Vitro Release Methods By An Optimized HPLC Method

Optimize Edilen HPLC Yöntemi İle L-DOPA Yüklü PLGA Nanopartiküllerinden Etkin Madde Salımının Farklı İn Vitro Çıkış Yöntemleri İle Değerlendirilmesi

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ABSTRACT

Objective: Dissolution tests have emerged in the pharmaceutical field as a very important tool to characterize drug product performance. To assure product quality and performance after the manufacturing process, changes in the formulation can be assessed through dissolution tests during the development of new formulations.

Methods: In this study, a simple High Performance Liquid Chromatography (HPLC) method was developed and validated for the quantitation of release of L-Dopa from poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles. Chromatographic separation was done with a reversed-phase C18 column, using acetonitrile-water containing 0,05% trifluoroacetic acid (5:95, v/v) as a mobile phase at a wavelength of 280 nm. The developed method was validated according to ICH guidelines for its specificity, linearity, accuracy, and precision.

Results: The method was shown to be linear ($r^2 \geq 0.995$) over the concentration range of 1,25 - 40 µg / mL. The mean % recoveries were in between, 102.59 % -98.70 % indicating an agreement between the true value and the detected value. Solution stability was guaranteed with antioxidant addition. The analytical method was shown to be suitable for the evaluation of L-Dopa which is released from nanoparticles. In vitro release studies were performed using sample and separate (SS), and dialysis membrane (DM) methods. Difference (f_1) and similarity (f_2) factors were used in the comparison of the SS and DM methods. The difference in the

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 Basıncılı Sıvı Kromatografisi (HPLC) yöntemi geliştirilmiş ve onaylanmıştır. 280 nm dalga boyunda 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, doğrusalığı, doğruluğu ve kesinliği için ICH kurallarına göre doğrulanmıştır.

Bulgular: Yöntemin 1.25 - 40 µg / mL konsantrasyon aralığında doğrusal ($r^2 \geq 0.995$) 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 yapılmıştır. SS ve DM yöntemlerinin karşılaştırılmasında farklılık (f_1) ve benzerlik (f_2) faktörleri kullanılmıştır. L-Dopa salım kinetiğindeki fark her iki yöntem için de anlamlı bulunmamıştır ($f_1 < 15$ ve $f_2 > 50$).

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L-Dopa release kinetics were not significant for both methods ($f_1 < 15$ and $f_2 > 50$).

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

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

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 a chronic disorder which has a progressive and neurodegenerative effect on the movement and expression responsible neurons, and can be defined as a progressive disease which affects dopaminergic neurons, especially nigro-striatal area and surrounding pathways. Recent data suggests that 1–3% of population in the age range over 65 years of all races is suffering from PD (1, 2). PD's conventional treatment is based on replacing dopamine in living neurons by administering L-Dopa which is the most commonly used dopamine precursor. Conventional therapy has some disadvantages such as fluctuant plasma levels of L-Dopa because of irregular gastric emptying and intermittent oral intake (3), poor relative bioavailability (~5–15%), and less than 1% of the administered dose reaching to the brain (4–7).

Polymeric nanoparticles are prepared with natural or synthetic polymers. In recent studies, the most commonly used nanoparticles produced from biodegradable and biocompatible polymers such as PLGA or poly(glycolic acid). Nanoparticles are preferred due to their ability to provide high chemical and biological resistance, to carry both hydrophilic and lipophilic active substances, and to be given to the body in different ways.

Nanoparticles release small amounts of encapsulated drugs as a function of time, analytical method must be very sensitive to quantify drug concentrations in the dissolution medium during in vitro release experiments. In vitro dissolution/release testing methods for nanoparticulate delivery systems can be broadly divided into three categories: *i) membrane diffusion methods (such as the dialysis methods) (DM); ii) sample and separation methods (SS); and iii) continuous flow methods.* DM, the most commonly used method to perform in vitro release studies from nanoparticles was chosen as reference method. SS, second common used method was chosen as test method in our study design.

Among the analytical methods currently available, HPLC is one of the most common analytical methods used for the characterization of pharmaceuticals. HPLC-diode array detector (DAD), offers a quick, sensitive and accurate method to separate and identify drugs in pharmaceutical nanoparticle formulations (8, 9). Although many researchers have investigated the in vitro drug release from nanoparticles, there aren't any known HPLC method for the simultaneous determination of L-Dopa has been developed and applied for the quantification of released L-Dopa

from nanoparticles in dissolution medium, with the help of DM and SS methods. Also, in most of the reported studies, L-Dopa solutions with low pH level were used as standard solution (10–13).

Nanoparticles were designed to be transported to brain via nasal route by endocytosis. To demonstrate the dissolution profile of L-Dopa from nanoparticles two buffered solutions that mimic endolysosomal compartment's and brain pHs (pH 4.5 and pH 7.4) were used (14). As mentioned before, literature studies about the determination of L-Dopa in acidic solutions provides information that L-Dopa is stable in acidic conditions (10, 12, 13, 15). Therefore, we optimized stability indicated analytical method to determine L-Dopa amount in different media which has higher pH values as difference to literatures.

Nanoparticles were prepared by double emulsion-solvent evaporation method, using methylene chloride as organic solvent, polyvinyl alcohol as surfactant and PLGA as the polymer in our work. In order to determine the release profile of L-Dopa from PLGA nanoparticles, SS and DM methods were compared. In vitro release method is thought to signify the influence of changes in the manufacturing procedure on the formulation to characterize drug product performance. So in vitro dissolution test methods used was compared and HPLC method validated to ensure the robustness of the analytical method developed, including stability tests of the L-Dopa.

METHODS

Instrumentation

Agilent 1100 series integrated HPLC system with DAD, pump, auto-sampler and degasser unit were used for this experiment. 250 mm × 4.6 mm, 5 μ reverse-phase C18 HPLC column was from Macherey-Nagel. Orbital shaker and ultra centrifuge (Sigma® 30KS) were used for in vitro drug release studies.

Reagents and materials

L-Dopa was gift from ILKO Pharmaceuticals (Ankara, Turkey). Deionised water was acquired from a Millipore water supplier. Trifluoroacetic acid (TFA), NaCl, Na₂HPO₄, KH₂PO₄, Acentonitrile (HPLC grade) and Tween 80 were from Merck. The mobile phase was filtered through a 0.45 μ m membrane filter (Millipore, Barcelona) and degassed with ultrasonic bath, prior to use. Dialysis tubing cellulose (14,000 MWCO) and Polypropylene co-Polymer centrifuge tubes were supplied by Sigma-Aldrich. Poly (vinyl alcohol) (PVA) were from Sigma-Aldrich.

Chromatographic system and conditions

Wavelength was selected based on absorbance maximization of the drug as per UV spectrum. 280 nm was found ideal for L-Dopa for HPLC analysis. Mobile phase was comprised of TFA solution (% 0.05 v/v) pH 3 - acetonitrile (95:5). 250 mm × 4.6 mm, 5 µm C18 HPLC column was used for chromatographic separation. Flow rate was 1 mL/min and run time was 7 minutes with 10 µL injection volume (1). The present method was developed and validated in consideration of the ICH guideline Q2 (R1) consisting the following parameters: specificity, linearity, detection and quantification limits, repeatability and intermediate precision, accuracy and stability (16).

Preparation of working solutions

Suitable analytical procedures should be applied for defining of the amount of L-Dopa dissolved during dissolution test. 100 µg/mL stock solution were prepared by dissolving a suitable amount of L-Dopa in buffer solutions pH 4.5 and pH 7.4. All solutions were sonicated for 30 min before use. 6 concentration point range of 1.25 - 2.5 - 5 - 10 - 20 - 40 µg/mL (ppm) was constructed by diluting from stock solutions with buffer solutions pH 4.5 and pH 7.4. For Stability of L-Dopa in diluents (phosphate buffer solution pH 4.5 and pH 7.4) was investigated for over 48 hours at different temperatures (4° C, 25 ° C, 37 ° C) in presence and absence of ascorbic acid.

Preparation of nanoparticles

L-Dopa loaded nanoparticles were prepared using a double emulsion solvent evaporation method using PVA as a stabilizer. Briefly, L-Dopa and PLGA were dissolved in dichloromethane (DCM), and distilled water was added to that solution and by means of using an ultrasonic homogenizer they were mixed to form a primary W/O emulsion. The primary emulsion was further emulsified in PVA solution by homogenization to obtain a double W₁/O/W₂ emulsion. The resultant double emulsion was then stirred at a certain rate at room temperature (25°C) on a magnetic stirring plate to evaporate the organic solvent. The resulting nanoparticle suspension was kept at 4°C overnight to allow hardening of the PLGA matrix by allowing the DCM to fully partition to the external aqueous phase. Nanoparticles were recovered by ultracentrifugation. The supernatant was removed and nanoparticles sediments were washed twice with distilled water to remove free drug and excess surfactant, and then lyophilized.

Dissolution test development

Dissolution medium

The term sink conditions is defined as the ability of the dissolution media to dissolve at least 3 times the amount of drug in the dosage form. Percentage of drugs released should be detected with the developed analytical method. Selection of the most suitable media condition is based on stability of the analyte in the test medium, and applicability to in vivo performance. In vitro release of L-Dopa from PLGA nanoparticles was performed in pH 4.5 to mimic the endolysosomal pH and pH 7.4 to mimic the brain pH as the nanoparticles were designed to target brain

with endocytosis after nasal administration (17). Phosphate buffer solution pH 4.5 was prepared as dissolving 6.8 g potassium dihydrogen phosphate, 2 % Tween80, 0.1 % ascorbic acid (aa) in 1000 mL distilled water and phosphate buffer solution pH 7.4 was prepared as dissolving 2.38g disodium hydrogen phosphate, 0.19g potassium dihydrogen phosphate, 8 g sodium chloride, 2 % Tween80, 0.1 % aa in 1000 mL distilled water. 2 % (w/v) Tween 80 was used to enhance the solubility of L-Dopa in aqueous solution. 0.1 % (w/v) ascorbic acid was added to protect L-Dopa from oxidation during study (18).

Method development

In vitro drug release (SS method)

L-Dopa loaded nanoparticles weighed to contain 45 µg L-Dopa were suspended in 5 mL of buffer solution. The suspension were poured in tubes and placed in an orbital shaker bath which was maintained at 37 ± 0.5 °C with a rotation of 100 rpm. At 30, 60, 120, 240 minutes, the tubes were taken out of the water bath and centrifuged 20 minutes at 2000 rpm. The precipitated nanoparticles were resuspended in 5 mL of fresh buffer (pH 4.5) and then put back in the shaker bath. The supernatant was taken for determination of L-Dopa concentration. All experiments were done in triplicate and results were given with variations.

In vitro drug release (DM method)

L-Dopa loaded nanoparticles; containing 45 µg L-Dopa were suspended in 0.5 mL of buffer solution and was inserted in a dialysis bag. The dialysis bag was put in 4.5 mL (total 5 mL) buffer solution at pH 4.5 and placed in an orbital shaker bath which was maintained at 37 ± 0.5°C with a rotation of 100 rpm. At 30, 60, 120, 240 minutes 1 mL aliquots were taken and replaced with 1 mL fresh pH 4.5 buffer. Drug concentrations were determined with the given analytical method. All experiments were done in triplicate. Results were given with variations.

Statistical calculations

All results are reported with mean and standard deviation of replicates. At all time intervals drug release (%) were discussed using two tailed t-test with Prism Software Version 6.0 a considering significant differences with $p < 0.05$. f_1 and f_2 factor were calculated by using Microsoft Office Excel®.

RESULTS

Mobile phase was comprised of TFA solution (% 0.05 v/v) pH 3 - acetonitrile (95:5). 250 mm × 4.6 mm, 5 µm C18 HPLC column was used for chromatographic separation. Flow rate was 1 mL/min and run time was 7 minutes with 10 µL injection volume. No interference was observed after enjection of L-Dopa solution, placebo and nanoparticles,

The regression equations for the calibration curve was found to be $y = 5.7424x + 2.1421$ (Figure 3) for phosphate buffer solution pH 4.5 and $y = 7.4159x + 9.5333$ (Figure 4) for phosphate buffer saline solution pH 7.4. The regression coefficient r^2 was 0.9998 for pH 4.5, 0.995 for pH 7.4 solution. Linearity data

was calculated with Prism Software Version 6.0 as shown in Table 1. RSD % of repeatability analysis in pH 4.5 buffer solution was between 3.07 % - 0.03 % . For pH 7.4 RSD % values were between 0.83 % - 0.01 % . The result revealed the precision with RSD %, 1.44% to 0.01 % for pH 4.5 and 1.00 % and 0.01 % for pH 7.4, respectively for inter day. The mean % recoveries were in between 102.59 – 98.70 for pH 4.5 and 101.92-100.00 for pH 7.4. L_D and L_Q values for pH 4.5 was found to be 0.0408, 0.1235 µg/ml and for pH 7.4 was found to be 0.0636 , 0.1928 µg/ml respectively. It was found that remaining L-Dopa was 97.46 % at 4° C, 95.54 % at 25° C, 89.63 % at 37° C for pH 4.5 solution in presence of ascorbic acid for 48 hours. It was found that remaining L-Dopa was 103.21 % at 4° C, 88.21 % at 25° C, 75.85 % at 37° C in pH 7.4 solution in presence of ascorbic acid for 48 hours.

The release rate differences were evaluated for SS and DM method. f_1 was found 6.02 and f_2 was found 95.38 At all time intervals drug release (%) was not different between test and reference methods ($p=0.853$; $p<0.05$).

DISCUSSION

Optimization of the chromatographic method

The HPLC method was developed for the determination of drugs was able to sustain a reliable quality control analysis. The wavelength was selected based on absorbance maxima of three different concentrations of L-Dopa solutions as per UV spectrum. A wavelength of 280 nm was selected because it provides high sensitivity which is needed for the quantitation of significantly low drug concentrations in the dissolution samples.

To determine the peak of L-Dopa, mobile phase has to have the

adequate polar:apolar solvent ratio. Some important parameters such as the pH of the mobile phase, percentage of organic modifier were tested for an acceptable chromatographic separation. Experiments were conducted with 1 mL/min flow rate, 7 minutes run time and 10 µL injection volume. 10 ppm concentration L-Dopa was given the system as sample. Experiments showed that acidic mobile phase with reverse phase C18 column provides symmetric and sharp peaks and provides high solubility for L-Dopa. Therefore TFA solution was determined as acidic buffer solution as shown Figure1. Zhou, Alany, Chuang, Wen ¹

When the ratio of mobile phase was formed with acetonitrile-0.05% (v/v) TFA solution (5:95 v/v) from (10:90, v/v) the peak of active substance was cracked. Acetonitrile-0.05% (v/v) trifluoroacetic acid solution (2.5 : 97.5 , v/v) ratio for mobile phase was tested for a better separation. However, peak of L-Dopa couldn't be determined quite contrary to our previous experiments. To protect the column's integrity, TFA solution was adjusted at pH 3 with 0.1 N HCl acid solution and peak tailing was acceptable (Figure 1).

The symmetry factors obtained for the sample peak (1.058) was determined in the limits <1.5 of various pharmacopeias (Figure 1). Therefore, the HPLC analysis parameters were settled as

Table 1. Linearity Data of 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 - 4.896
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

Figure 1. Preliminary chromatogram with C18 phase

Table 2: Repeatability analysis of the assay in pH 4.5 buffer solution for HPLC (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

250 mm×4.6 mm, 5 μ C18 HPLC column for chromatographic separation with a rate of 1 mL/min flow with 7 minutes run time, 10 μ L injection volume, 25°C column temperature and acetonitrile-0.05% (v/v) TFA solution (5:95, v/v) (pH 3) for mobile phase (1).

Nanoparticles were designed to be transported to brain via nasal route by endocytosis. To demonstrate the dissolution profile of L-Dopa from nanoparticles two buffered solutions that mimic endolysosomal compartment's and brain pHs (pH 4.5 and pH 7.4) were used (14). As mentioned before, literature studies about the determination of L-Dopa in acidic solutions provides information that L-Dopa is stable in acidic conditions (10, 12, 13, 15). Therefore, we optimized stability indicated analytical method to determine L-Dopa amount in different media which has higher pH values as difference to literatures. In addition, pH 4.5 and pH 7.4 buffer solution with and without L-Dopa are injected to determine whether there is interference in the peaks. Any interference was observed (Figure 2).

Method validation

The present method was developed and validated in accordance with the ICH guideline Q2 (R1)(16). The most widely applied validation characteristics; linearity, accuracy, precision, specificity, stability, detection limit and quantification limit were performed. The method was then utilised to quantificate of released drug amount from nanoparticles containing L-Dopa.

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

Linearity

Linearity was studied by preparing standard solutions at different concentration levels. ICH guidelines specify a minimum of five concentration levels, linearity was determined for L-Dopa in the range of 1.25 - 40 μ g/mL at six concentration levels by performing three independent repetitions (n=3) (16). Samples were analysed six times per concentration; peaks areas were documented, analysed and results of the regression statistics are given in Figures 3 and 4. A good linear relationship ($r^2 \geq 0.995$) was observed between the concentrations and the respective peak areas provided by the detector (Figures 3&4). The regression coefficient $r^2 \geq 0.995$ considered as evidence of acceptable fit

Figure 3: Calibration curve of L-Dopa in pH 4.5 buffer solution for HPLC (n=3)

Figure 4: Calibration curve of L-Dopa in pH 7.4 buffer solution for HPLC (n=3)

Table 3: Repeatability analysis of the assay in pH 7.4 buffer solution for HPLC (n=6)

1.25 μ g/mL		5 μ g/mL		20 μ g/mL	
Area	Concentration μ g/mL	Area	Concentration μ g/mL	Area	Concentration μ g/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

of the data to the regression line according to the ICH guidelines (16). Acceptability of linearity data is often judged by examining the correlation intercept. Also slope of line should be calculated for better analysis (Table 1) (8). These results show us that all calibration curves have suitable linearity according to ICH guidelines (16).

Precision

The absolute difference values between the mean assay results obtained from the repeatability and intermediate precision tests meet the acceptance criteria (% relative standard deviation (RSD)<2.0). Repeatability, establishes the precision of multiple sampling under the same operating conditions over a short interval time, while intermediate precision establishes variations from the same laboratory under variable operation conditions, for example different days, analysts or equipment. Hence, the method was found precise (19).

Repeatability

Repeatability prove precision under the same operational conditions over a short period of time. Repeatability is also attributed to as intra-assay sensitivity. In this study; low (1.25 µg/mL), medium (5 µg/mL) and high (20 µg/mL) concentrations representing the calibration curve were analyzed to determine mean, standard deviation (SD) and RSD (n=6). RSD % for standart peak must be lower than 2.0 (20). The results in Tables 2&3 show that the method meets the requirements of this analytical validation parameter for medium (5 µg/mL) and high (20 µg/mL) concentrations (20). Replication of 1.25 µg/

mL which represents the lower concentration, %RSD was higher than 2.0. However, there are also a number of literature that extend the limits to 5-6 % (8). The concentration 1.25 µg/mL represents the lowest point of calibration curve, so extension of limits seems suitable for this method.

Intermediate precision

The analyst variations of the method were determined using replicate injections of the concentrations given below and analyzed with different analysts on the same day (Tables 4&5). Intermediate precision was performed by RSD % of six repetated assays on samples at three concentration levels. RSD % values were found for all concentration levels and pHs to be 1,44 % and 0,1 %. For intermediate precision, the RSD % value should not exceed 2.0 % The results in Tables 4 and 5 show that the method meets the requirements of this analytical validation parameter (16).

Accuracy

The accuracy was determined by recovery of known amounts of L-Dopa. For the purpose of testing the accuracy of analytical methods, three concentrations (1.25; 5; 20 µg/mL; the low, medium, high concentrations, respectively) were diluted from stock solutions, covering the linear range of analytes were used. The mean % recoveries were in between 101.92-98.70 % implying an agreement between the true value and the found value (Table 6-7). All results meet the requirements for method verification according to the ICH guidelines (16).

Table 4: Precision analysis of the assay in pH 4.5 buffer solution for HPLC (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

Table 5: Precision analysis of the assay in pH 7.4 buffer solution for (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

Table 6: Accuracy analysis of the assay in pH 4.5 buffer solution for HPLC (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

Specificity

Specificity can be defined as the analyte being assessed precisely in the presence of expected components. This definition has a descriptive effect on the identity of an analyte. In our study, specificity tests were performed using three different polymers prepared nanoparticles and L-Dopa in two media. It was decided that the HPLC method was specific for the determination of L-Dopa according to the data obtained in Table 8. L-Dopa samples were injected five times and the similar retention times were observed in all cases. All prepared nanoparticles with different polymer substances were injected with or without L-Dopa. Any interference was not observed between the peaks of nanoparticles and L-Dopa. As a result the described HPLC method was specific for pH 7.4 and selective for pH 4.5 solutions.

Stability

The stability of L-Dopa in aqueous solution was evaluated to verify that if any spontaneous degradation occur when the samples were prepared (21). The aqueous solutions of L-Dopa

were found to be unstable. However, aqueous solutions have been proven to be stable in the presence of high concentrations of acidic substances. Stability of L-Dopa was not affected by light. The storage condition for the active substance was specified as 2-8°C. In addition, it has been proven that in the presence of antioxidants, L-Dopa can be protected from oxidation. Therefore, we planned to obtain information about the stability of L-Dopa in the presence of ascorbic acid in the body and during the validation and formulation studies (3, 18, 22). 0.4 mg/mL L-Dopa containing stock solutions were prepared with pH 4.5 and pH 7.4 buffered solutions. The solutions were mixed and vortexed until solid particles disappeared, then separated into 3 mL aliquots. The stability of the solutions containing ascorbic acid was extended up to 48 hours. Any oxidation was not observed in aa containing solutions at 4°C. As a result of the study, it is clarified that ascorbic acid should be added as a preserving agent in dissolution medium to perform the dissolution studies. Interference was not observed between peak of L-Dopa and ascorbic acid. The results are given in Table 9-10.

Table 7: Accuracy analysis of the assay in pH 7.4 buffer solution for HPLC (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

Table 8. The results of specificity analysis of HPLC method 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

Table 9. Stability study results of L-Dopa in pH 4.5 solution (aa= ascorbic acid)

	pH 4.5			aa not added		
	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

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 of three fold of the baseline-to-noise. Limit of quantification (L_Q) represents as lowest concentration level that is provided by a peak area with signal-to-noise. Calibration curve was calculated by using concentrations in the range of 1.25 - 40 $\mu\text{g/mL}$. With following equations;

$$\text{Detection limit } (L_D) = 3.3 \text{ a/s;}$$

$$\text{Quantification limit } (L_Q) = 10 \text{ a/s;}$$

where a is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve. For L_D and L_Q , where there isn't any defined limit in literature, are specific for each method. As shown in Table 12, obtained results meet our requirements for in vitro dissolution tests (23).

In vitro drug release

To design the dissolution media for a poorly soluble drug, the first method is increasing the volume of aqueous sink conditions or decreasing the dissolved drug amount. The addition of anionic or non-ionic surfactants and solubilizing the drug by co-solvents up to 40 % is another used approach. The other approach is to

alter the pH for a better sink condition. The last two approaches seem less cumbersome and have been more widely employed in pharmaceutical dissolution tests. 2 % Tween 80 was used to enhance the solubility of L-Dopa and ascorbic acid was added to the used mediums to protect L-Dopa from 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 released from nanoparticles for both methods. To achieve sink conditions L-Dopa concentrations shouldn't exceed 20 % of its saturation solubility in dissolution medium like in this study. So the reason for inhibited release was not insufficient sink conditions. Also this was not the case because of the inadequate loading of nanoparticles (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 for discussion of both methods. In vitro release profiles of the test formulations were similar with the reference formulation. In literature, it is told that release rate of the encapsulated drug is progressively higher when SS method compared with the DM (Figure 5) (24). This might be due to differences in

Table 10. Stability study results of L-Dopa in pH 7.4 solution (aa= ascorbic acid)

pH 7.4						
	aa added	aa not added	aa added	aa not added	aa not 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

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

	L_D	L_Q
pH 4.5	0.0408	0.1235
pH 7.4	0.0636	0.1928

Table 12. f_1 and f_2 statistics for the dissolution profile using the 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

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

the hydrodynamics of the system when compared with the nanoparticles in the dialysis bag to the formulation dispersed in a flask (25).

The similarity factor (f_2) is a measurement of the similarity in the percent (%) dissolution between the two profiles (26). The difference factor (f_1) is proportional to the average difference between the two profiles. FDA guide proposes that $f_1 < 15$ and $f_2 > 50$ to indicate equivalence in dissolution profiles (27).

$$f_2 = 50 \times \log \left(\left[1 + \frac{1}{n} \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \times 100 \right)$$

$$f_1 = \left[\frac{\sum_{t=1}^n (R_t - T_t)}{\sum_{t=1}^n R_t} \right] \times 100$$

As shown in Table 12, the difference in the L-Dopa release kinetics was not significant for both methods ($f_1 < 15$ and $f_2 > 50$) (24, 28). Comparison of the dissolution profiles was held with the two tailed t-test. At all time intervals drug release (%) was not different between test and reference methods ($p=0.853$; $p < 0.05$).

CONCLUSION

The validated methods were found to be simple, sensitive, accurate and precise. The results indicated that these methods are suitable for dissolution studies as well as routine quality control analysis of L-Dopa in nanoparticulate formulations. DM and SS methods gave similar in vitro release profiles at acceptable precision ($<10\%$, SD). The SS method tended to show faster release rates from formulations than those observed using dialysis bag (Table 12). The difference between DM and SS methods in the release kinetics was considered not significant ($f_1 < 15$ and $f_2 > 50$). The optimal method with respect to in vivo relevance in light of an in vivo in vitro correlation will need to be determined for further studies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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