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Brain Targeted Delivery of Levetiracetam Loaded Nanosphere

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Abstract: Designing an effective intranasal (IN) delivery mechanism for the water-soluble anti-epileptic drug levetiracetam (LEV) for brain targeting effect was the main objective of this work. By using the nanoprecipitation process and the polymer Eudragit S100, nanospheres were prepared. Different weights of Eudragit S100 and varied concentrations of poloxamer 188 (stabilizer) were employed. The produced levetiracetam nanospheres exhibited sufficient entrapment efficiency range from 79.2% to 93.5%, with zeta potential values from 26.7 mV to 40.6 mV. The developed Nanospheres had spherical shape and nanosize range (10.44 to 79.07 nm). In situ gels prepared from Poloxamer 407 (18%) and different mucoadhesive polymers (sodium carboxymethylcellulose (Na CMC) and chitosan) were evaluated for gelling time, gelling temperature, pH and rheology. The nanosphere in situ gels were further evaluated for in vitro drug release and revealed 73.6% to 84.5% release within 8 hrs. The optimum in situ gel formula, based on rank of rheology and % release after 8 hrs, was evaluated for stability, and evaluated for ex-vivo permeation through nasal mucosa.

Keywords: levetiracetam, nanosphere, Nanoprecipitation, brain targeting, Eudragit S100, intranasal, in situ gel.

1. INTRODUCTION

Epilepsy is considered one of the most prevalent neurological illnesses, affects more than 50 million people worldwide, and each year approximately 5 million new cases are diagnosed. Epilepsy is characterized by aberrant brain activity resulting in convulsions. Depending on basic brain dysfunctions, this neurological disorder comprises numerous etiologies including abrupt and excessive neuronal discharges that result in epileptogenesis. Although it affects people of all ages, the rate of disease is high in children and elderly persons [1]. Levetiracetam (LEV) is an antiepileptic medication with acceptable pharmacologic properties and proven effectiveness in enhancing seizure control. LEV exhibits no marked affinity for benzodiazepine or gamma-aminobutyric acid (GABA) receptors, according to in vitro tests. LEV works in the brain through a unique binding site and novel mechanism. The synaptic vesicle protein, a crucial membrane protein found on synaptic vesicles, is this novel binding site [2]. As LEV is highly water soluble and has no effective permeation through BBB, a novel delivery system to enhance its permeability through blood brain barrier (BBB) is needed. Since the beginning of the twenty-first century, nanotechnology with a wide range of nanocarriers such as liposomes and nanoparticles focused on targeted drug delivery has been expanding quickly [3]. Nanoparticles (NPs) are one of the nanosystem delivery methods which have been developed to accomplish extended or organized drug delivery, to increase the bioavailability, drug stability, and drug targeting to the site of action [4]. Nanospheres are small particles with a size range of 10 to 200 nm. The hydrophobic surfaces of these particles are susceptible to opsonization and clearance by the reticuloendothelial system. Nanospheres can be prepared by various methods but solvent evaporation is the most widely used. Nanospheres are extensively used for targeted delivery systems. Polyacrylates and poly-

methylmethacrylates (marketed as Eudragit®) are examples of non-biodegradable polymers. These polymers have an affinity for matrix disintegration in the body, making them useful in a variety of targeted drug delivery system [5]. The Intranasal route of administration is an established strategy for delivering medications that have either a local effect or a systemic one, and it has excessive interest due to its distinctive brain–nose structural connection. Intranasal administration is a non-invasive technique that delivers medications to the brain (bypassing the blood–brain barrier) along the olfactory and trigeminal neural pathways, limiting systemic exposure and side effects.

Indeed, the olfactory and trigeminal nerves, located in the olfactory and respiratory region respectively, allow for the nose-to-brain absorption of drugs aimed acting at the central nervous system (CNS) level [6]. As a result of interactions between the components of gel that are activated by either physical causes such as pH, temperature or chemical factors for example oxidative reactions, in situ gelling systems, the solution to gel conversion occurs after being inserted into the nasal cavity [7]. Hydrogels responsive to temperature variation are one of the most broadly investigated environment-sensitive drug delivery systems and particularly, those based on poloxamers have been extensively studied to obtain in situ forming nasal gels [8].

Poloxamers are water soluble consisting of a unit of Poly Propylene Oxide which is a hydrophobic part surrounded by two units of Polyethylene Oxide as hydrophilic part. The ability to form ordered structures in solution known as micelles due to both hydrophilic and hydrophobic units allow for encapsulation of hydrophobic and hydrophilic medicines. When the temperature rises from the temperature of polar solution to temperature of crucial micelle, the Poly Propylene Oxide chains lose solubility of the formed micelle and tangle before gel formation.

Poloxamers have also been extensively used as good additives in medicines with a variety of applications, including targeting the central nervous system and delivery systems of drug. Poloxamers are FDA-accepted as well as included in the American and European Pharmacopoeia [9]. Polymers such as Na CMC and Chitosan, which have mucoadhesion effect, are used with Poloxamer to give mucoadhesion and enhance the residence in the nasal cavity because poloxamer by itself typically can't provide this effect [10].

2. METHODS

2.1. Material:

Levetiracetam (LEV) was supplied by Hekma pharm for pharmaceulical industry (Egypt) as asample gift**.** Eudrgit S100, Poloxamer 188, poloxamer 407 and Na CMC were supplied by Egyptian International Pharmaceutical Industries co. (EPICO), Cairo, Egypt. Methanol, disodium hydrogen phosphate and potassium dihydrogen phosphate, El- Nasr Pharmaceutical Co. (Egypt). Chitosan was supplied from Sigma Aldrich (Germany). Cellulose membrane was bought from Sigma Aldrich., Germany.

2.2. Establishment of standard curve:

A solution of 100 µg/mL of levetiracetam (LEV) in Phosphate Buffer Solution (PBS) (pH 6.4) was prepared as stock solution. Aliquots of 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.4, 2.6 and 2.8 mL were separately put in a suitable measuring flask (10 mL) then diluted with PBS (pH 6.4) to produce a concentration of 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 µg/mL, respectively. The absorbance was measured spectrophotometrically at the predetermined λmax (210 nm) using PBS (pH 6.4) as a blank [13]**.**

2.3. Preparation of nanosphere formulations containing levetiracetam*:*

Nanospheres were formed by the nanoprecipitation method. In an eppendorf tube of 2 mL, the drug (25 mg) and eudragit S100 were correctly weighed at different weights (50, 75, and 100 mg) and were dissolved in 2 mL methanol, because it is a water-miscible solvent using an ultrasonic bath for duration of 10 min. then this organic solution was dropped (0.5 mL/min) into 8 mL deionized water which contained poloxamer 188 (stabilizer) in various concentrations (0.5, 1 and 1.5%), using magnetic stirrer at a speed of 500 rpm. Spontaneously, nanospheres were precipitated then converted into a colloidal dispersion. By continuous stirring for 1 h, the remaining organic solvent evaporated. Lastly, to facilitate size reduction, the nanospheres dispersion was put in an ultrasonic bath and sonicated for duration of 30 min [14]**.**

2.4. Characterization of nanospheres:

2.4.1. Particle Size, Polydispersity Index (PDI) and Zeta Potential:

The Particle Size (PS), zeta potential, and Polydispersity index (PDI) of nanosphere were measured using dynamic light-scattering technique (DLS) in the zetasizer nano (**Malvern , UK)**. PS and

PDI of NPs prepared were measured by dispersing the formulation in distilled water. Zeta potential values were evaluated by using the same equipment in a single used capillary zeta cell at 25 °C and dissolved in deionized water. Measuring of all samples was done three times for statistical analysis [15] .

2.4.2. Drug entrapment efficiency:

The centrifugation approach, which includes separating the drug-loaded nanospheres from the free drug solution, was used to determine the concentration of drug entrapped in nanospheres (aqueous phase). The concentration of free LEV present in the supernatant was measured in order to assess the entrapment efficiency. Therefore, one milimeter of formulation was taken then centrifuged at high speed (15,000 rpm) at 4°C for duration of 30 min. An Ultraviolet-visible spectrophotometer was used to measure the LEV concentration in the supernatant at 210 nm. [16 -17].

This equation was used to detect entrapment efficiency (%) of LEV:

Entrapment efficiency (%) = [(Mass of initial LEV - mass of free LEV)/mass of initial LEV] × 100%.

2.4.3. Transmission electron microscope (TEM):

The optical observation of the formulation was done by spreading a tiny amount of sample which has been

2.4.4. Differential scanning calorimetry analysis (DSC):

DSC studies were carried out for LEV, Eudragit s100, a physical mixture diluted on glass slide then shielded with a coverslip. The method used for TEM (PHILIPS-CM 200), SAIF, IIT Bombay, Mumbai) measurement was the negative staining method. By staining a drop of sample using phosphotungestic acid, deposited on copper micronets then allowed to dry at 25°C before being examined under the microscope lens [17- 18].

2.4.5. Differential scanning calorimetry analysis (DSC):

DSC studies were carried out for LEV, Eudragit s100, a physical mixture of LEV with Eudragit s100 and the selected nanosphere formulation (**Schimadzu, model DSC-50, Japan**). Under nitrogen condition, the samples had been heated to 300 ˚C at the rate of 10 ˚C /min.

2.4.6. Fourier transforms infrared spectroscopy analysis of Eudragit S100 nanospheres (FTIR):

Using an FT-IR spectrometer, (FTIR) analysis was done to detect any chemical interactions between the LEV and ES100 (**Perkin-Elmer, FTS-1710, Beaconsfield, UK**). LEV, ES100, physical mixture of LEV with ES100, and selected nanosphere formulation had been examined by FTIR spectrometer in the region of 4000–400 cm^{-1}[19].

2.5. Preparation of levetiracetam nanosphere mucoadhesive in situ nasal gel:

The selected nanosphere formulation containing levetiracetam, equivalent to 10 % w/v of levetiracetam, and mucoadhesive polymer (Na CMC, and chitosan) in concentration of 0.5, 1 and 1.5% were sprinkled in distilled H_2o on magnetic stirrer at 25 ºC. Then the formed solution was maintained at 4Cº overnight for complete hydration of polymers. After cooling, 18% w/v of Poloxamer 407 was sprinkled gradually on magnatic stirrer. After the dispersion had developed, it was left at a refrigerator temperature 4°C overnight till the formed solution being clear and transparent. Last of all, the volume of the formed gel had been adjusted to 100 mL with dist water [20, 21]. For the chitosan-containing formulations, the chitosan was dissolved in 1mL of 0.1N acetic acid and processed as directed in the technique of preparation [22].

2.6. Evaluation of levetiracetam mucoadhesive in situ nasal gels:

2.6.1. Determination of pH:

From each formulation, 1 mL gel sample equivalent to 100 mg of drug was transferred to the 10 mL volumetric flask and diluted with distilled water. The digital pH meter was standardized using a buffer of pH 4 and a buffer of pH 7 then the pH of resulting solutions was determined [23].

2.6.2. Determination of gelation temperature and gelation time:

In a test tube, a measured amount (2 mL) of all gel formulations were transmitted and covered with parafilm then dipped in a water bath. The water bath temperature was raised slowly at a rate of 1°C/ 2 min beginning with 25°C to the temperature at which gel formed. Then each sample was evaluated for gelation [23]. Measurements were done three times.

The test tube inversion method was used to assess the gelation time. One mL of the gelling solution (sol) was placed within test tube in a thermostatically controlled water bath maintained at 37 °C. By angling the tube every 10 seconds until no flow was detected, the transition time was calculated [21].

2.6.3. Rheological study:

Using an MCR502 rheometer, the rheological characteristics of gel formulations were examined at 35°C (nasal temperature). The Shear rate was raised from 1 to 100 S^{-1} progressively. Examining the viscoelastic characteristics of hydrogel formulations can be done with the help of rheological measurements. Thus, in order to assess the shear rate as a function of shear stress, continuous shear experiments were carried out in the tested formulations [21; 24].

2.6.4. Evaluation of the In vitro release of in situ nasal gel formulations containing levetiracetam loaded nanosphere:

The study of in-vitro release of nanosphere in situ gel formulations was through cellophane membrane using fabricating diffusion cell. The cellophane membrane had been soaked in PBS (pH 6.4) overnight. Then the membrane was fixed at one side of a glass diffusion cell. One mL of gel formulation was placed in donor compartment, and then the diffusion cell put in 300 mL of Phosphate buffer solution of pH 6.4 as receptor compartment using temperature of 35±0.5°C and keeping rotation speed at 50 rpm [25]. Volumes of 3mL were taken out from the receptor compartment within time intervals of 1,2,3,4,5,6,7, 8 and 24 hr and replaced with 3 mL of fresh PBS of pH 6.4 at each time interval to maintain the sink condition. The concentration of LEV released was calculated using UV Spectrophotometer at λ_{max} 210 nm. Experiment was done in triplicate.

2.6.5. Selection of the optimum formulation:

Ranking of different prepared in situ gel formulations was carried out based on their rheological study and percent release of drug after 8 hours. The selected formulation (optimum) is the one that exhibited high viscosity at low shear rate (10 s-1) and had farrow's constant value (N) between one and five [37]. Regarding percent release of drug, the selected formulation released about 80% of drug along eight hours [40]. The optimum in situ gel formulation which was selected after ranking of prepared formulations according to rheological study and percent release of drug after eight hours was subjected to stability study and ex - vivo study through sheep nasal mucosa.

2.6.6. Stability study:

Stability study of the optimum LEV nanosphere loaded nasal in-situ gel was conducted at room temperature (25ºC) and refrigerator temperature (4ºC) in closed containers for 6 months. Following that, the optimised batch was evaluated for drug content and gelation temperature at intervals of 0, 1, 2, 3, and 6 months [26, 27].

2.6.7. Ex vivo study:

From the fresh nasal tissue of sheep which gotten from a local slaughterhouse, nasal mucosa had been isolated within 1 hour of the sheep butchering. The isolated nasal mucosa was cleaned from any Fatty tissues or other tissues. The uncontaminated nasal mucosa was maintained in a saline solution at a temperature of 20 °C. Nasal mucosa was mounted between the receptor and donor chambers. Volume from optimum LEV nanosphere loaded in situ gel and plain LEV loaded gel equivalent to 100 mg was retained in direct contact to the mucosa in the donor medium. The receptor medium was 25 mL of phosphate buffer pH 6.4 at 50 rpm and kept at 37 °C. Sink condition is the ability of the dissolution media **to dissolve at least 3 times the amount of drug that is in your dosage form**. Levetiracetam is freely soluble in water (1.04g/1ml). The volume of dissolution medium (phosphate buffer solution pH 6.4) was 25 mL which is considered more than the volume required for complete solubility of drug and guarantee sink conditions achievement. Samples were withdrawn at intervals untill 12 hr and were substituted with equivalent volume of Fresh Phosphate Buffer pH 6.4. The absorbance of each sample was measured at 210 nm [26].

Table 1. The composition of nanosphere formulations.

Formulations	Eudragit S100 (mg)	Poloxamer 188 (%)	Levetiracetam (LEV) (mg)
NS1	50	0.5	25
NS2	75	0.5	25
NS3	100	0.5	25
NS4	50	1	25
NS5	75	1	25
NS6	100	1	25
NS7	50	1.5	25
NS8	75	1.5	25
NS9	100	1.5	25

3. RESULTS

3.1. Estimation of Standard calibration curve of levetiracetam in PBS (pH 6.4):

At the predetermined λ_{max}, the standardization curve of the LEV in Phosphate Buffer Solution (pH 6.4) was linear (R^2 = 0.9961) over the concentration range 2- 28 µg /mL. It was clear that the solution of levetiracetam obeys Beer's Lambert law within the tested concentrations of LEV at predetermined λ_{max} 210 nm. The absorption of all samples had been

plotted against the corresponding concentration as shown in figure (1).

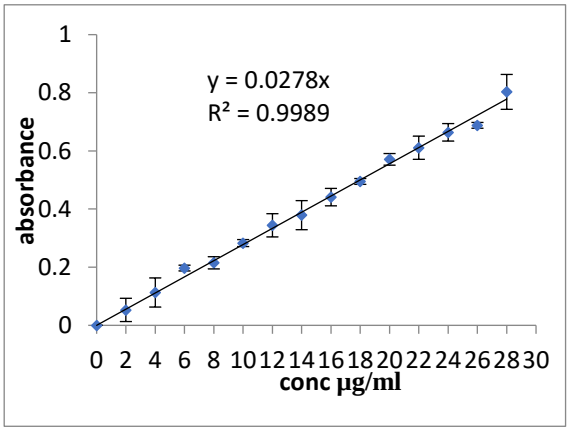


Figure 1. Calibration curve of levetiracetam in PBS pH (6.4) at λ_{max} 210 nm.

3.2. Evaluation of the nanosphere formulations:

3.2.1. Particle size, polydispersity index and zeta potential of nanospheres:

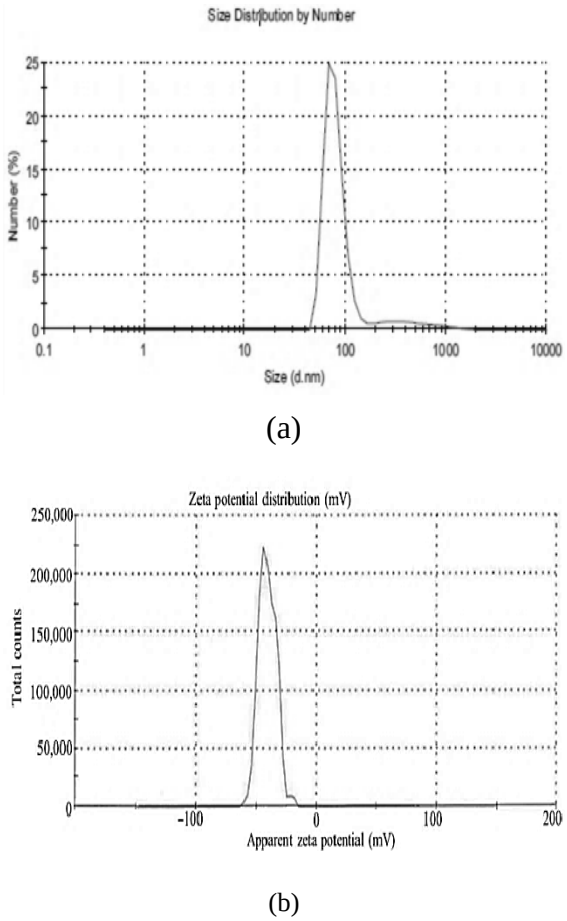
Table (3) displays the results of particle size, polydispersity index and zeta potential. Particle size of all formulations **ranged from** 10.44±0.11 **to** 79.07±0.7nm. Zeta potential values are ranged from -26.7±2.1 to -40.6±0.14.

3.2.2. Drug entrapment efficiency percent:

Table (3) shows the EE% for all formulations. Entrapment efficiency range was from 79.2±0.18 to 99.74±0.85.

Table 2. Particle size, PDI, zeta potential and entrapment efficiency percent of perepared nanosphere formulations.

Formulations	PZ (nm) ± SD	PDI± SD	ZP (mV) ± SD	%EE± SD
NS1	10.44±0.11	0.462±1.3	-26.7±2.1	79.2±0.18
NS2	20.28±0.5	0.465±0.8	-29.9±3.1	89.7±0.14
NS3	67.58±0.8	0.306±1.5	-25.9±1.1	97.4±1.02
NS4	13.67±1.2	0.207±2.1	-31.2±0.09	81.2±2.01
NS5	24.48±0.9	0.283±0.85	-34.5±0.14	85.6±2.12
NS6	74.25±1.6	0.325±0.1	-36.3±0.42	98.1±1.06
NS7	16.17±0.6	0.25±1.7	-37.9±0.12	85.5±0.5
NS8	62.72±1.2	0.455±1.8	-37.8±0.45	93.5±0.14
NS9	79.07±0.7	0.108±1.4	-40.6±0.14	99.74±0.85



(a)

(b)

Figure 2. (a) particle size and (b) zeta potential of selected nanosphere formulation (NS9).

3.2.3. Transmission electron microscope (TEM) of nanosphere:

TEM was employed in studying the morphology of the selected nanosphere formulation (NS9) after it was placed on a copper grid. The TEM investigation revealed that the nanospheres were all spherical in shape, had a smooth surface, and had a uniform size scattering. The particle size of the nanospheres was detected to be in the nanometer range (76.6 nm).

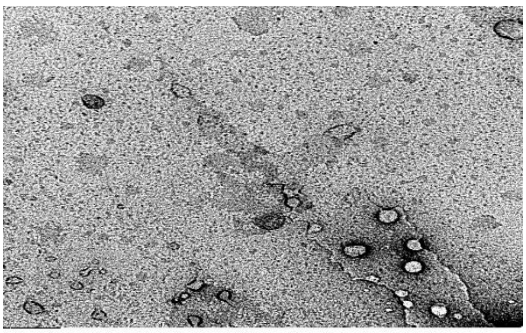


Figure 3. Transmission Electron microscope of the selected nanosphere formulation (NS9).

3.2.4. Differential scanning calorimetry analysis (DSC) of LEV loaded nanosphere:

Levetiracetam DSC thermograms show a single endothermic peak at 119.3 °C, which corresponds to the melting point of LEV [32]. While the DSC thermogram of eudrgit S100 shows endothermic peak at 70°C [33]. In the DSC thermogram of levetiracetam – eudragit S100 physical mixture there are the two endothermic peaks of levetiracetam and eudragit S100 with little shift indicating the compatibility between LEV and ES100. No melting peak of LEV appeared in the DSC thermogram of the selected nanosphere formulation (NS9) and this indicates that LEV was entrapped in nanospheres well[19] .

3.2.5. Fourier transforms infrared spectroscopy (FTIR) analysis of Eudragit S100 nanospheres:

Figure (5) illustrates the FTIR spectra of levetiracetam alone. It exhibits NH stretching at 3360 cm^{-1}, CH stretching at 2897 cm^{-1}, CO bending at 1681 cm^{-1}, CH bending at 1431 cm^{-1}, and CN stretching at 1082 cm^{-1} as its absorption bands [34]. The FTIR spectrum of eudragit S100 shown a broad OH absorption band at 3257 cm^{-1}, methyl and methylene CH stretching vibrations at 2997 and 2961 cm^{-1}, C=O stretching at 1732 cm^{-1}, and C–O–C stretching vibration at 1157 cm^{-1} [35]. In the FRIR spectrum of the selected nanosphere formulation (NS9), it was observed the absence of all characteristic peaks of the LEV and this indicates that the LEV was encapsulated in the nanosphere [19].

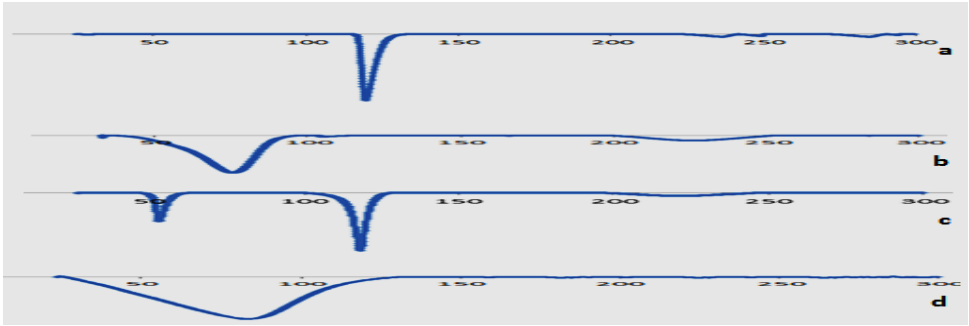


Figure 4. DSC thermograms of a) free LEV, b) ES100, c) LEV and ES100 physical mixture and d) selected nanosphere formulation (NS9).

Table 3. Composition and Evaluation of levetiracetam mucoadhesive in situ nasal gels:

Formulations	NaCMC (%)	Chitosan (%)	Distilled Water to	0.1N acetic acid (mL)	Drug content % ± SD	Gelation temperature °C± SD	Gelation time (sec) ± SD	pH ± SD
NG1	0.5		100		99.1 ± 0.44	32 ± 0.75	24 ± 0.14	6.3 ± 0.06
NG2	1		100		98.2 ± 0.32	30.3 ± 0.5	21 ± 0.32	5.4 ± 0.03
NG3	1.5		100		100.1 ±0.52	28.6 ± 0.51	18 ± 0.41	5.06 ± 0.05
NG4		0.5	100	1	98.17 ± 0.53	34 ± 0.6	23 ± 0.3	4.04 ± 0.1
NG5		1	100	1	99.04 ± 0.87	32 ± 0.45	19 ± 018	4.14 ± 0.05
NG6		1.5	100	1	100.2 ± 0.59	30 ± 0.64	15 ± 0.21	4.8 ± 0.04

***all formulations contain 18% poloxamer 407.**

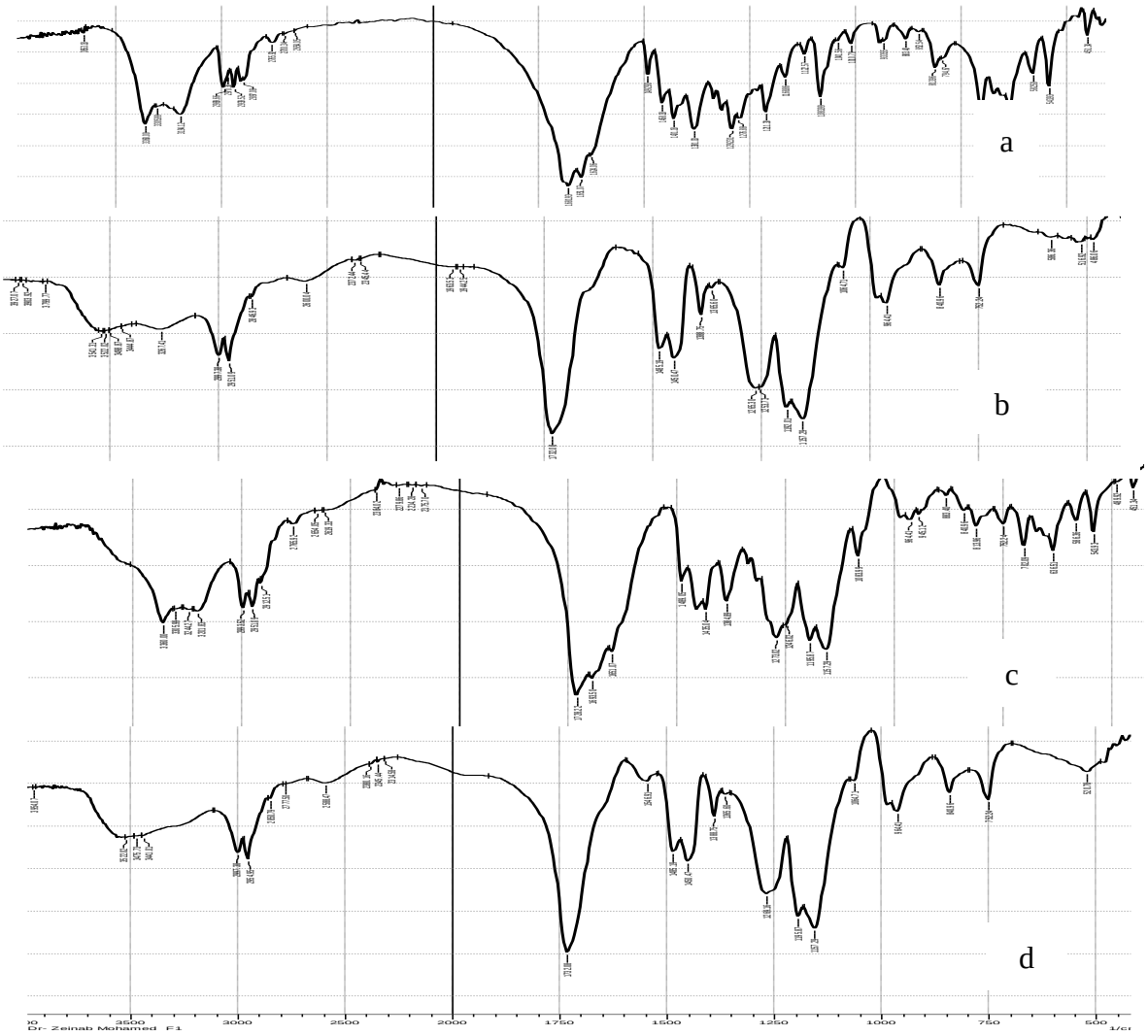


Figure 5. FTIR spectra of (a) LEV, (b) ES100, (c) LEV and ES100 physical mixture and (d) the selected nanosphere formulation (NS9).

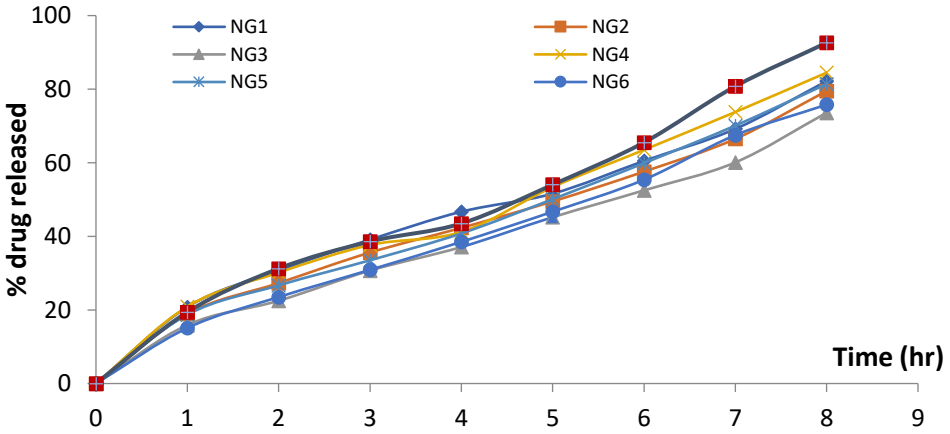


Figure 6. Percent LEV released from in situ nasal gel formulations containing LEV loaded nanospheres and selected nanosphere.

3.3. Results of evaluation of levetiracetam mucoadhesive in situ nasal gels:

3.3.1. pH:
From table (4), it is obvious that pH of all formulations (NG1 to NG6) are in the range from 4.04 ± 0.1 to 6.3 ± 0.06 which indicates suitability of the formulations for nasal administration [25].

3.3.2. Gelation temperature and gelation time of in situ nasal gels:
The results of gelation temperatures of all in situ gel formulation showed in table (4). The temperature of gel formation was observed between (28.6°C ± 0.51 and 34°C ± 0.6) for the batches (NG1 to NG6). It was found that the time required to formation of gel for in situ gel formulations ranges from 15 ± 0.21 to 24 ± 0.14 sec, which is considered a suitable time [6].

3.3.3. In-vitro release of levetiracetam from in situ nasal gel formulations containing LEV loaded nanospheres:

After 8 hours, the cumulative proportion of LEV released by all gel formulations varied between 70% and 85%. Within the first hour, all formulations showed an initial burst release and after that a controlled release.

3.3.4. Rheological studies:
Regarding the rheograms for the medicated gel formulations, all of them exhibited shear-thinning and pseudoplastic behavior (table 6 and figure 7).

Selection of the
According to results of rheological study and percent release of drug after eight hours, formula NG2 showed viscosity of 43152 mpa. s at shear rate 10s-1 with Farrow's constant (N) of 1.838 and released 79.5% of drug after eight hours, thus this formula was selected for further stability and ex-vivo studies. **These results are obtained in table (7).**

3.3.5. Stability study:
After being stored at refrigerator temperature (4°C) and room temperature (25°C), there are no significant changes discovered in the formulations (table 8).

3.3.6. Ex-vivo permeation study of the optimum LVT loaded in situ nanosphere gel:
The % LEV permeated from LVT loaded nanosphere in situ gel and the plain LVT in situ gel were 75.5 and 67.8, respectively (figure 8).

4. DISCUSSION:

The main benefits of choosing the nanoprecipitation method over other ones for creating polymeric nanoparticles are its simplicity and speed of production, high reproducibility, limited exposure to and use of organic solvents by the maker, affordability, and simplicity of scaling-up. By using this technique, a milky-looking suspension of nanoparticles was produced when the organic phase containing LEV dissolved in methanol was added to the aqueous solution containing Poloxamer 188. As a result of the organic phase's spontaneous dispersion in the aqueous solution and the solvents' miscibility, opalescence systems were created [5].

It has been proven that eudragit S100 weight changes have an impact on the nanosphere particle size. It was detected that the particle size of nanosphere formulation increases by increasing the polymer weight from 50 mg to 100 mg. This is due to increase in viscosity of the polymer solution which leads to reduced diffusion of polymer into the aqueous phase [28].

The particle size of the nanospheres dramatically rises when the concentration of Poloxamer 188 is increased from 0.5% to 1.5%, as was shown when comparing each formula to its equivalent that contained more poloxamer content. This may be explained by the accumulation of poloxamer molecules within the formed nanosphere as well as their surfaces. Increased poloxamer chain density per unit volume can produce more massive structures that produce bigger particles [29].

Good homogeneity and a limited size distribution are confirmed by PDI [27]. For the resulting dispersion to be stable, zeta potential is necessary. Zeta potentials of the formed nanospheres fell in the range from -26.7±2.1 to -40.6±0.14 mV. The Negative sign of zeta potential is due to the anionic polymer, Eudragit S100, as a result of the appearance of free carboxyl groups at the polymer extremities [30].

Increases in Eudragit S100 were seen to result in an increase in EE%. This could be as a result of ES100 concentration increasing the viscosity of the organic phase, which increasedthe barrier to drug diffusion from the organic to the aqueous phase and increased encapsulation efficiency [28]. Poloxamer 188 as well showed a like effect on EE% and a raise in concentration of Poloxamer 188 lead to increasing of entrapment efficiency; this may be due to creation of an interaction between the hydrophobic units of Poloxamer 188 and Eudragit during nanoprecipitation process [31].

Table 4. Farrow's constant and mean apparent viscosity of the in situ gel formulations:

Formulations	Farrow's constant (N)	Mean apparent viscosity (mPa . s)		
		at shear rate 10 S^{-1}	at shear rate 50 S^{-1}	at shear rate 100 S^{-1}
NG1	12.737	27697	6320.4	2365
NG2	1.838	43152	8805.3	3531.5
NG3	7.145	81366	7186.5	2106.7
NG4	4.787	25981	11682	7577
NG5	4.19	28787	5690.6	2696.8
NG6	1.94	39361	6895.6	4796.9

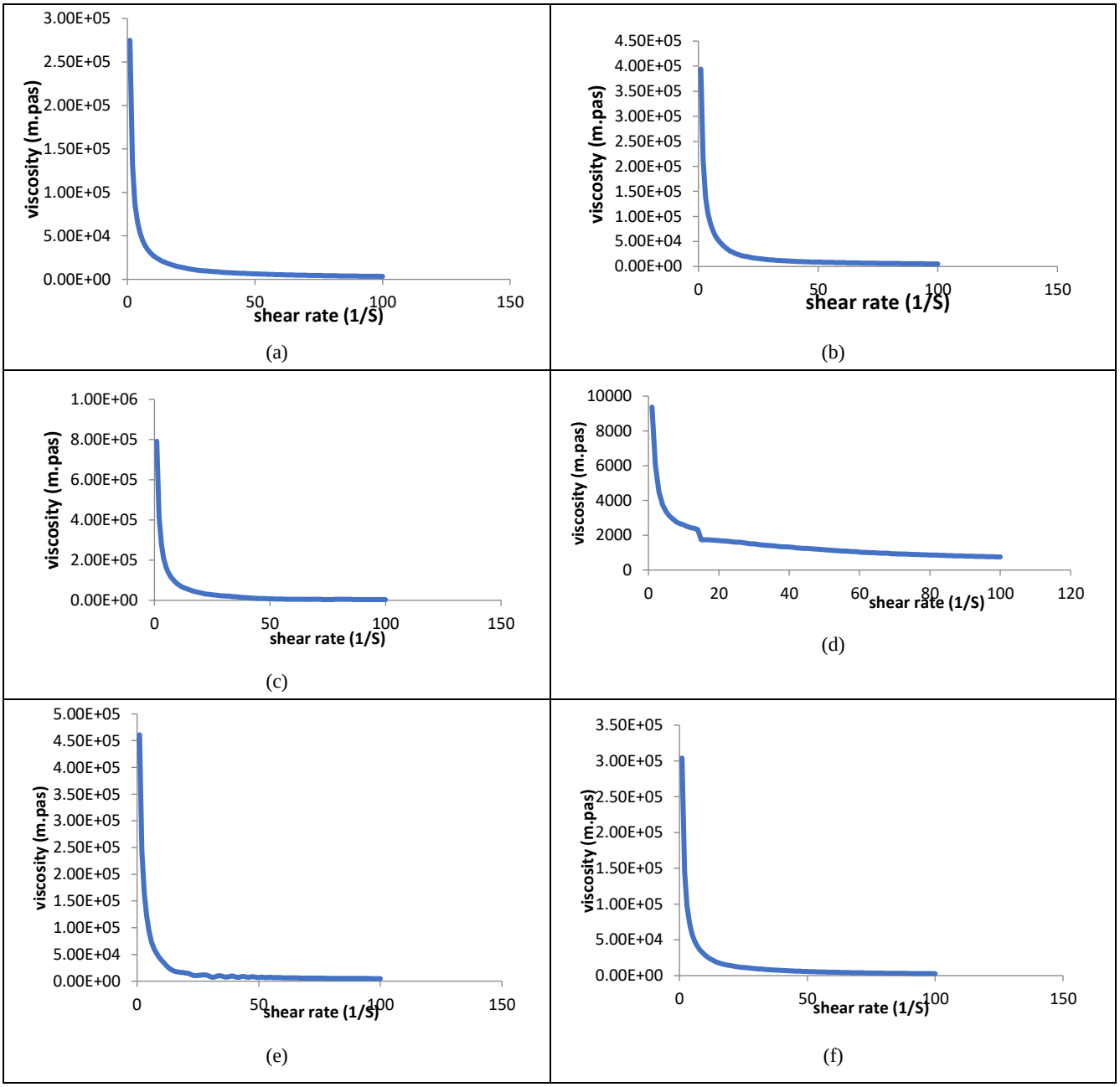


Figure 7. Relation between the dynamic viscosity and shear rate of (a) NG1, (b) NG2, (c) NG3, (d) NG4, (e) NG5 and (f) NG6

Table 5. reveals ranking of prepared in situ gel formulation according to rheology and % release within 8 hrs**.**

Formulations	Farrow's constant (N)	Rank (N)	% release (8 hr)	Rank % release	Viscosity At shear rate 10 s-1	Rank Viscosity	Total rank
NG1	12.737	6	82.2	2	27697	4	12
NG2	1.838	1	79.5	2	43152	2	5
NG3	7.145	5	73.6	4	81366	1	10
NG4	4.787	4	84.5	3	25981	4	11
NG5	4.19	3	81.4	1	28787	4	8
NG6	1.94	2	75.8	3	39361	3	8

Table 6. The stability assessment

	Time (month)	**Drug content (%)**	**Gelation temperature ºC**
At 4ºC	0	98.2 ± 0.32	30.3 ± 0.5
	1	98.1± 1.01	30.5 ± 0.32
	2	97.9 ± 0.25	30.8 ± 0.25
	3	97.7 ± 1.04	30.7 ± 1.03
	6	95.6 ± 1.02	30.9 ± 2.01
At 25 ºC	0	98.2± 0.32	30.3 ± 0.5
	1	97.5± 1.01	30.6 ± 1.02
	2	97.1 ± 1.2	30.9 ± 1.2
	3	95.9 ± 0.25	31.5 ± 1.04
	6	91.9 ± 1.05	32.1 ± 1.4

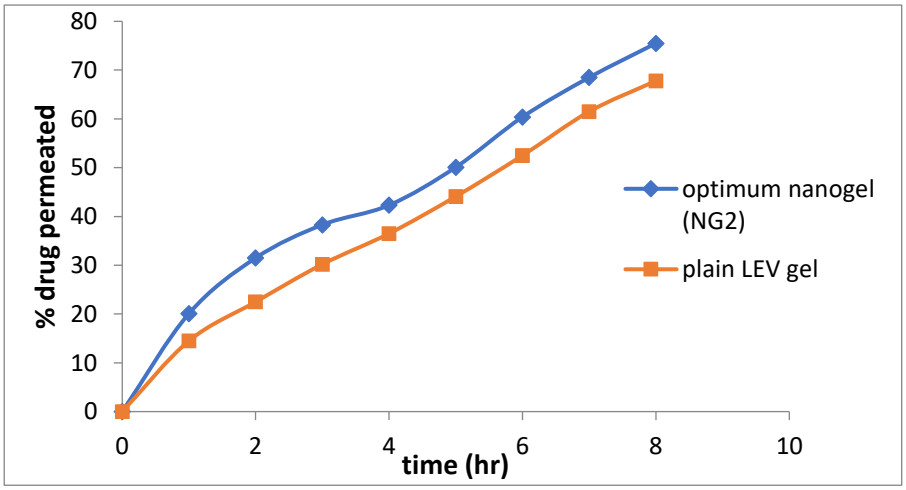


Figure 8. Percent drug permeated from optimum nanogel (NG2) and plain LEV gel through sheep nasal mucosa.

In the formation of hydrogels, sodium carboxymethylcellulose (Na CMC) is one of the most often used polymers. Its high water absorption capacity, minimal immunogenicity, and excellent biocompatibility with skin and mucous membranes have made it a popular gel-forming polymer[11].

Being a cationic polysaccharide generated from chitin, chitosan is biodegradable and biocompatible. This polymer has considerable versatility because, although being insoluble in water, the chitosan structure can be changed without degrading its physical, chemical and biological characteristics [12].

In in situ formulations, the temperature at which the gel formed has a critical character in in situ gels. By activation of any stimuli (physical or chemical factors) such as temperature, solution is transformed into gel and this is the gelation temperature. The gelation temperature below 25°C produce administration problems and more than 37°C can lead to rapid escape from the nose. The perfect temperature for formulation to be applied in the nose is between 28 and 32 ^{o}C because the nose temperature is 35 ^{o}C [10]. It was detected that LEV release was decreased when the LEV loaded nanospheres was incorporated in a gel structure. This is may be due to that LEV need to be released from nanospheres into the hydrogel then diffused from the gel structure to the released medium [36].The results of percent drug released were presented in figure (6).

It was found that the primary viscosity (at 10 s^{-1} shear rate) increased when the polymer concentration increased. At room temperature gel formulations were liquid and obtained rapid gelation when temperature increased [37]. To determine the effect of polymer concentration and shear rate upon gel viscosity, the in situ gel viscosity was measured with respect to shear rate at 10 s^{-1}, 50 s^{-1}, and 100 s^{-1}. At refrigerator temperature, the gel formulation's gelation temperature did not significantly change. Also, when compared to formulation held at ambient temperature, a greater drug concentration was discovered when stored in a refrigerator. Thus, it was discovered that refrigeration was preferable to room temperature for the storage of LEV-loaded In-situ gel [38].

The permeation study was used to evaluate the effect of the gel structure on LEV nanosphere diffusion through the nasal membrane. And from the ex-vivo study we can expect the permeability of LEV when administrated in vivo. The results of permeation of LVT loaded nanosphere in situ gel in comparison with the plain LVT in situ gel were studied.

The nanoscale of the nanosphere particles is the primary reason for the permeability improvement. It was detected that the Particle size which is < 500 nm aids the nanospheres to pentrate through non-aqueous pores present in the mucous membrane. The particle size significantly affected the transport of LEV nanosphere across the blood brain barrier. Regarding the composition, poloxamer 188 is copolymer (surfactant) which has the ability to increase permeability of LEV, accordingly the penetration through the mucosal membrane increases. The slow release exhibited by the two formulations may be due to the incorporation of LEV and the LEV-loaded nanospheres within the in situ gel structure which formed when exposure to the phosphate buffer solution (PBS) [39].

5. CONCLUSIONS:

The present study shows that LEV loaded nanospheres prepared successfully by nanoprecipitation technique. Formulations were evaluated with several parameters. The prepared nanospheres have a spherical morphology with negative zeta potential. The selected optimum formulation (NS9) shows acceptable nano-sized particles (79.07nm) with low PDI value (0.108), zeta potential (-40.6 mV), and high entrapment efficiency (99.7%). NS9 was further incorporated in a temperature triggered in situ gelling system. Optimum LVT loaded nanosphere in situ gel (NG2) showed all physical characters within the limit that satisfy the need of use. In stability study, NG2 provide physicochemical stability for six months. The ex-vivo study showed an improved penetration of the LEV loaded nanosphere across the sheep nasal mucosa from gel. From this study results, it was indicated that the potential use of current LEV loaded nanosphere gel for LEV transport systems through nose and formula NG2 is a promising candidate for further evaluation in a clinical trial.

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List of Abbreviations: IN: Intranasal, **LEV**: Levetiracetam, **NaCMC**: sodium carboxymethylcellulose, **BBB**: blood brain barrier, **NPs**: Nanoparticles, **CNS**: central nervous system, **PS**: Particle size, **PDI**: Polydispersity index, **TEM**, Transmission electron microscope, **DSC**: Differential scanning calorimetry analysis, **FTIR:** Fourier Transform infrared, **ES100**: Eudragit s100, **PBS:** Phosphate buffer solution, **EE%**: entrapment efficiency percent

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