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Original Article

Nanoemulsion formulation containing carbamazepine and levetiracetam: Development and *in vitro* characterization

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ABSTRACT

Background and Aims: Epilepsy is one of the most disabling and most common neurological disorders, affecting approximately 65 million people of all age groups worldwide. When there is no response to monotherapy in the treatment of epilepsy, the combined use of Carbamazepine (CARBA) and Levetiracetam (LEV), which have different mechanisms of action, can be additive/synergistic and may be useful in the clinic. The aim of our study is to develop a fixed-dose combination (FDC)-nanoemulsion (NE) formulation containing CARBA and LEV for the treatment of epilepsy.
Methods: Blank NE (BLNK-NE) and CARBA+LEV-FDC-NE formulations were prepared and carried out the *in vitro* characterization studies [morphological analysis, centrifugation test, droplet size (DS), polydispersity index (PDI), zeta potential (ZP), viscosity and pH measurements, FT-IR analysis, the percent entrapment efficiency (EE%), and *in vitro* release study].
Results: The DS, PDI, pH, and ZP values for the BLNK-NE formulation were found to be 117.63±3.82 nm, 0.240±0.014, 4.62±0.03, and (-)26.07±3.04 mV, respectively. For the CARBA+LEV-FDC-NE formulation, the DS, PDI, pH, and ZP values were determined as 137.56±3.11 nm, 0.225±0.013, 4.60±0.06, and (-)21.62±0.29 mV, respectively. The EE% values obtained for CARBA+LEV-FDC-NE were 97.33±0.56% (for CARBA) and 96.73±0.91% (for LEV).
Conclusion: The CARBA+LEV-FDC-NE formulation was successfully prepared. This formulation had suitable *in vitro* characterization results.
Keywords: Carbamazepine, epilepsy, fixed-dose combination, levetiracetam, nanoemulsion

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INTRODUCTION

Epilepsy, one of the most disabling and most common neurological disorders affecting approximately 65 million people of all age groups worldwide, is characterized by an enduring predisposition to produce recurrent and unprovoked seizures or electrical disturbances in the brain and associated psychological, cognitive, and social consequences (Devinsky et al., 2018; Kanner & Bicchi, 2022; Patel & Parikh, 2020). The sudden appearance of temporary signs and symptoms caused by excessive and abnormal or synchronized neuronal activity in the brain is defined as an epileptic seizure (Kanner & Bicchi, 2022). There are three diagnostic levels (seizure type, epilepsy type, and epilepsy syndrome) in "the International League Against Epilepsy 2017 Classification". It is also emphasized that etiology and comorbidities should be considered at each level (Wirrell, Tinuper, Perucca, & Moshé, 2022). The most common types of epilepsy are focal and generalized epilepsies (Kanner & Bicchi, 2022). Epilepsy treatment approaches include using antiepileptic drugs (AEDs), a special diet (ketogenic diet), vagus nerve stimulation, or surgery (Green, Nguyen, Kaalund-Hansen, Rajakulendran, & Murphy, 2020). AEDs should be selected according to epilepsy and seizure types, epilepsy syndrome, and drug-related adverse effects. The main goal of epilepsy treatment with AEDs is to eliminate seizures while minimizing AED-related adverse effects (Kanner & Bicchi, 2022).

Carbamazepine (CARBA), a derivative of dibenzazepine used for the treatment of epilepsy, trigeminal neuralgia and pain associated with other neurological disorders, is a sodium channel blocker and also modulates other voltage-gated ion channels (e.g., voltage-gated calcium channels) (Beydoun et al., 2020; Maan, Duong, & Saadabadi, 2022; Uzunović, Vranić, & Hadžidedić, 2010). Dizziness, nausea, vomiting, ataxia, and drowsiness are the most common side effects associated with using CARBA. Also, a few severe skin reactions are rarely seen (Maan et al., 2022). CARBA is a potent cytochrome P-450 enzyme inducer and is significantly more prone to drug-drug interactions (Beydoun et al., 2020). It is a BCS (The Biopharmaceutics Classification System) Class II active substance (low solubility and high permeability), and its oral bioavailability is limited by its dissolution rate. Therefore, the solubility and dissolution rate of CARBA are critical determinants of its oral bioavailability (Uzunović et al., 2010).

Levetiracetam (LEV) is a second-generation established AED, approved by the FDA in 2000 for adjunctive therapy in treating myoclonic seizures, focal seizures, and primary generalized seizures (Kumar, Maini, & Kadian, 2022; Lyseng-Williamson, 2011). It is thought that LEV exerts its effect by binding to a unique synaptic vesicle protein 2A (SV2A is found on synaptic vesicles and some neuroendocrine cells), thereby regulating the release of neurotransmitters, including excitatory amino acids and ultimately suppressing epilepsy discharge (Yi et al., 2018). LEV is very soluble in water and shows high permeability. Therefore, LEV is a BCS Class I active substance (Petruševska et al., 2015). LEV is rapidly absorbed and has a very high oral bioavailability (96%). The main metabolic pathway for LEV is the enzymatic hydrolysis of the acetamide group. LEV does not show a significant drug-drug interaction (pharmacokinetically) due to the fact that the hepatic cytochrome P450 system plays a minimal (2.5%) role in its metabolism (Kumar et al., 2022; Lyseng-Williamson, 2011). The most common side effects associated with LEV use are headaches, fatigue, depression, mood swings, sedation, agitation, irritability, confusion, nausea, vomiting, anorexia, abdominal pain, etc. (Kumar et al., 2022). In the treatment of epilepsy, when seizures cannot be controlled with monotherapy, combination therapy can be used for the patient (Sarhan, Walker, & Selai, 2016). There are two approaches to drug combination therapy: 1. the combined use of two or more drugs, which are used separately in the treatment, and 2. the use of a single dosage form containing at least two active substances (known as FDC) (Kim & Weon, 2021; Rahman et al., 2020). The primary purpose of treatment with FDCs is to increase therapeutic efficacy and improve patient compliance through synergistic effects while minimizing adverse effects/toxic effects (Kim & Weon, 2021). Although the efficacy of the combination therapy is supported by the data obtained, there is a possibility of increased side effects (Sarhan et al., 2016). In combination therapy, drug interactions (pharmacokinetic and pharmacodynamic interactions) should also be considered (Park, Kim, & Lee, 2019). For these reasons, patients should be followed carefully during combination therapy. In a review on the effect of antiepileptic drugs in combination therapy, it was reported that the combinations of LEV-oxcarbazepine or CARBA-LEV or lacosamide-LEV were the most common dual AEDs combinations (Mäkinen, Rainesalo, Raitanen, & Peltola, 2017).

In this study, we aimed to develop an FDC-NE formulation containing CARBA and LEV (CARBA+LEV-FDC-NE) for the combination therapy of epilepsy and carried out *in vitro* characterization studies of the NE formulations [BLNK-NE (without active substances) and CARBA+LEV-FDC-NE].

MATERIALS AND METHODS

LEV, CARBA, and Labrasol were generous gifts from DEVA Holding A.Ş. (Türkiye), Biofarma İlaç San. Tic. A.Ş. (Türkiye) and Gattefosse (France), respectively. Ethyl oleate and Tween 80 were obtained from Merck (Germany).

Methods

Preparation of BLNK-NE and CARBA+LEV-FDC-NE

The preparation method developed by Sigward et al. (2013) was modified and used in our study. For the preparation of FDC-NE formulations containing CARBA and LEV, the oil phase was prepared by mixing ethyl oleate (40%), surfactants [Tween 80 (35%) and Labrasol (15%)], and CARBA (200 mg) by first using a magnetic stirrer followed by Ultra-Turrax (T10, IKA, Germany). Besides, the solution of LEV (50 mg) in ultrapure water (as the aqueous phase) was prepared. The prepared aqueous phase was added to the oil phase to obtain the primary emulsion while mixing at high speed (27500 rpm). The primary emulsion [W/O(water/oil)] was added to ultrapure water (1:2 ratio) under magnetic stirring (750 rpm) and mixed until W/O/W (water/oil/water) NE containing CARBA and LEV (CARBA+LEV-FDC-NE) was obtained.

The procedure mentioned above was used for the preparation of BLNK-NE without active ingredients.

In vitro characterization of BLNK-NE and CARBA+LEV-FDC-NE

Centrifugation test

To assess the stability of BLNK-NE and CARBA+LEV-FDC-NE under stress conditions, we centrifuged the formulation samples (5 g each) at 3500 rpm (15 min.). After centrifugation, we evaluated the NE formulations for phase separation, creaming, or active substance precipitation.

The morphology of CARBA+LEV-FDC-NE

The CARBA+LEV-FDC-NE formulation was diluted 100-fold. Later, it was placed on a copper grid and dried at room temperature for 24 h. Then, we obtained the transmission electron microscope (TEM) images of the CARBA+LEV-FDC-NE using a Hitachi TEM HT7700 (Hitachi HighTech, Japan) operated at an accelerating voltage of 120 kV.

Measurements of the DS, PDI, ZP, pH and viscosity of BLNK-NE and CARBA+LEV-FDC-NE

The DS, PDI, and ZP values of BLNK-NE and CARBA+LEV-FDC-NE were determined at room temperature using ZetaSizer Nano ZSP (Malvern Inc., UK) at a scattering angle of 173°. The NE formulations were diluted 100-fold before the measurement.

In addition, the pH and viscosity values of these formulations were determined at room temperature using a pH meter (Thermo Scientific, Orion 3 StarTM, USA) and a viscometer (Brookfield RV DV2T, USA), respectively.

FT-IR analyzes of the active substances, BLNK-NE and CARBA+LEV-FDC-NE

We recorded the FT-IR spectra (4000-400 cm^{-1}) of CARBA, LEV, BLNK-NE and CARBA+LEV-FDC-NE using the Shimadzu IRSpirit-T model FT-IR spectrophotometer (Japan).

Determination of EE% for CARBA+LEV-FDC-NE

CARBA+LEV-FDC-NE formulation (0.25 g) was weighed, and the volume was made up to 10 mL with methanol in a volumetric flask (n=6). The mixture was stirred under a magnetic stirrer for 15 min. Then, the mixture was filtered through a membrane filter [PTFE, 0.45 µm (Agilent Captiva)]. The CARBA and LEV contents in the filtrate were analyzed using the HPLC method developed and validated in our previous study (Kandilli, Uğur, Çetin, & Miloğlu, 2018).

In vitro release study

In vitro release study was performed in two different release media (HCl pH 1.2 or phosphate buffer pH 6.8) using the dialysis bag method. The NE formulation (1 mL)-containing dialysis bag (MWCO 14.000 Da) was placed in a colored vial containing 100 mL release medium in a horizontal shaking water bath (37±0.5 °C, 50 rpm). 1 mL of sample was taken from the release medium at the specified time intervals (0.5, 1, 2, 3, 4, 8, 12, and 24 h), and the same volume of fresh release medium (warmed at 37±0.5°C) was added to maintain the constant volume (to maintain "Sink condition"). The samples were filtered through a membrane filter [PVDF, 0.45 µm, Isolab]. The CARBA and LEV contents in the filtrate were analyzed using the HPLC method developed and validated in our previous study (Kandilli et al., 2018).

Statistical analysis

We used the independent-t test (SPSS Statistics 22.0; SPSS Inc., Chicago, USA) to compare the results obtained from our study and found the difference between the two independent groups. $p<0.05$ was considered "statistically significant".

RESULTS AND DISCUSSION

NEs composed of water, oil, and surfactant/s are kinetically stable systems (Nastiti et al., 2017; Ugur Kaplan et al., 2019). Due to their small droplet size, usually in the range of 20-500 nm (Gupta, 2020), NEs show long-term physical stability (Rehman, Akram, Seralin, Vandamme, & Anton, 2020). NE has drawn increasing attention for its great potential to enhance the solubility of poorly water-soluble active substances and improve their oral bioavailability. In addition, it is a beneficial dosage form that combines different advantages such as ease of preparation, being prepared to contain both hydrophilic and hydrophobic active substances, and protecting the relevant active substance/s from environmental conditions (Rosso et al., 2020; Sabjan, Munawar, Rajendiran, Vinoji, & Kasinathan, 2020). There are studies on the preparation of NEs for the combinational delivery of two active substances, but these are commonly for cancer treatment (Alkhatib, Bawadud, & Gashlan, 2020; Alkreathy et al., 2020; Mahajan & Patil, 2021; Pangeni, Choi, Jeon, Byun, & Park, 2016).

In the literature, there are studies on the NE formulations containing CARBA (Echeverri et al., 2020; Kelmann, Kuminek, Teixeira, & Koester, 2007; Prokhorov et al., 2014) and a microemulsion formulation containing LEV (Djekic et al., 2021). However, there is no study on a NE formulation containing LEV and CARBA (in combination).

In our study, an FDC-NE formulation containing CARBA and LEV (active substances, one hydrophobic and the other hydrophilic) was developed, and *in vitro* characterization studies were carried out. In this way, we aimed to use two active substances with different action mechanisms in combination and to achieve an improvement in the solubility and oral bioavailability of CARBA.

In the centrifugation test, phase separation, creaming, or active substance precipitation was not observed in the NE formulations. The TEM images of CARBA+LEV-FDC-NE are shown in Figure 1. The DS, PDI and ZP values of BLNK-NE and CARBA+LEV-FDC-NE are given in Table 1.

DS, PDI, and ZP measurements are essential to assess the physical stability of NEs. In NE, which is a colloidal dispersion, the Brownian motion of small droplets contributes to resistance to physical destabilization caused by creaming, gravitational separation, and coalescence (Mahamat Nor, Woi, & Ng, 2017; Santos, Trujillo-Cayado, Carrillo, López-Castejón, & Alfaro-Rodríguez, 2022). Also, smaller droplets have a larger surface area, which provides a larger contact area with the intestinal mucosa, and as a result, NEs increase the absorption of the active substance (Hu, Xie, Zhang, Qi, & Li, 2021). In our study, the DS values of the NE formulations were in the nano-range (Table 1). The TEM images of CARBA+LEV-FDC-NE also supported this

result (Figure 1). Moreover, the droplets of CARBA+LEV-FDC-NE were approximately spherical in the TEM images (Figure 1). There was a significant difference ($p<0.05$) between the DS values of the BLNK-NE and CARBA+LEV-FDC-NE. The presence of active substances caused a slight increase in the DS of NE. The PDI values of the NE formulations were <0.3 (Table 1). This shows that the droplet size distributions of the NEs are in a narrow range and acceptable (Uğur Kaplan et al., 2019). The ZP values of BLNK-NE and CARBA+LEV-FDC-NE were about (-)26 mV and (-) 22 mV, respectively (Table 1). The difference between the ZP values of these formulations was significant ($p<0.05$). The change in the ZP value could be due to the chemical structure of CARBA and LEV. In a study, the effects of active substances with different physicochemical properties on the nanoemulsion formulation were investigated and compared to blank nanoemulsion with neutral ZP. It was stated that the ZP value of the nanoemulsion formulation containing cyclosporine A, which has an amine group in its structure, shifted slightly to positive, while the nanoemulsion containing curcumin with a hydroxyl group had a negative value (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). ZP values greater than or equal to (+) 30 mV and less than or equal to (-) 30 mV provide good physical stability for emulsions (Mahamat Nor et al., 2017). However, ZP values around (+/-) 20 mV can provide sufficient physical stabilization in the formulations where electrostatic stabilization is combined with steric stabilization. Non-ionic surfactants such as Tween 80 contribute to the physical stabilization of NEs by decreasing interfacial tension and providing a steric barrier against coalescence (Ugur Kaplan et al., 2019).

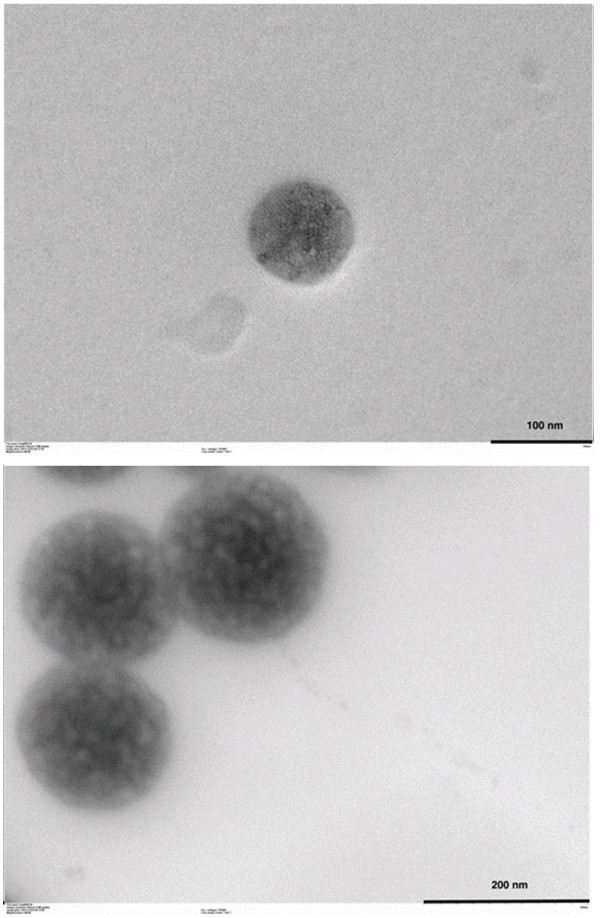

Figure 1. The TEM images of CARBA+LEV-FDC-NE.

pH is a critical parameter to be determined during the preparation of aqueous liquid formulations because it can affect the solubility and activity of the active substance, as well as the stability and biological tolerability of the formulation (Vázquez-Blanco, González-Freire, Dávila-Pousa, & Crespo-Diz, 2018). In a study, it was determined that CARBA was more stable under acidic conditions (Rajadhyaksha, Jain, & Amin, 2007). In another study, it was reported that LEV formulations prepared in the "Ora-Sweet and Ora-Plus" vehicle could remain stable for 91 days when they were stored at either 25°C (at pH 4.25) or 4°C (at pH 4.34) (Ensom, Decarie, & Rudolph, 2011). In our study, the pH value for both BLNK-NE and CARBA+LEV-FDC-NE was about 4.6 (Table 1). The difference between the pH values of both formulations was not significant ($p>0.05$).

FT-IR analysis can be performed to determine the presence of any interaction between the active substance/s and other formulation components. Therefore, we carried out FT-IR analysis in our study and gave the FT-IR spectra of active substances and the NE formulations in Figure 2. In this study, the reobtained FT-IR spectra for the active substances (CARBA and LEV; Figure 2) are compatible with the FT-IR spectra of CARBA, and LEV presented in our previously published paper (Kandilli et al., 2020). Moreover, the FT-IR spectra of BLNK-NE and CARBA+LEV-FDC-NE formulations given in Figure 2 were similar, and the peaks related to CARBA or LEV did not appear in the FT-IR spectrum of CARBA+LEV-FDC-NE. Consequently, there was no interaction between the formulation components with the active substances (CARBA and LEV). Also, CARBA and LEV dispersed in NE formulation at the molecular level.

The EE% values obtained for CARBA+LEV-FDC-NE were found to be 97.33±0.56% (for CARBA) and 96.73±0.91% (for LEV).

Figures 3-a and 3-b show the *in vitro* release profiles of CARBA and LEV from CARBA+LEV-FDC-NE in the two different release media (HCl pH 1.2 and phosphate buffer pH 6.8). In HCl pH 1.2,

Table 1. The DS, PDI, ZP and pH values of BLNK-NE and CARBA+LEV-FDC-NE (Mean±SD; n=9).

Formulation	DS (nm)	PDI	ZP (mV)	pH
BLNK-NE	117.63±3.82	0.240±0.014	(-)26.07±3.04	4.62±0.03
CARBA+LEV-FDC-NE	137.56±3.11	0.225±0.013	(-)21.62±0.29	4.60±0.06

SD: Standard deviation; BLNK-NE: Blank nanoemulsion; CARBA+LEV-FDC-NE: CARBA and LEV-containing fixed-dose combination nanoemulsion

the percent of CARBA released from NE formulation at 30 min and 24 h were about 9% and 99%, while the percent of LEV released from NE formulation at 30 min and 2 h were about 80% and 100%, respectively (Figure 3-a). In phosphate buffer (pH 6.8), the percent of CARBA released from NE formulation at 30 min and 24 h were about 9% and 97%, while the percent of LEV released from NE formulation at 30 min and 2 h were about 81% and 100%, respectively (Figure 3-b). According to these results, the pH of the release medium has no effect on the release of CARBA and LEV from the NE formulation. LEV, a hydrophilic active substance, was released more rapidly than the NE formulation, but CARBA, a hydrophobic active substance, was released more slowly than the NE formulation. This result obtained for CARBA may be due to its limited diffusion into the aqueous phase (external phase) of the emulsion (Echeverri et al., 2020).

Formulation components (such as water, oil, and surface-active agent/s) and their concentrations affect the viscosity of NE formulations. For example, when the surfactant content in the formulation decreases, the viscosity of the formulation increases, while increasing the water content in the formulation can cause a decrease in the viscosity (Lovelyn & Attama, 2011) Table 2 and Figure 4 show the viscosity values and rheograms of the BLNK-NE and CARBA+LEV-FDC-NE formulations, respectively. The difference between the viscosity values of the BLNK-NE and CARBA+LEV-FDC-NE formulations was significant ($p<0.05$). The presence of active substances (CARBA and LEV) in the formulation caused a slight increase in the viscosity of the NE formulation (Table 2).

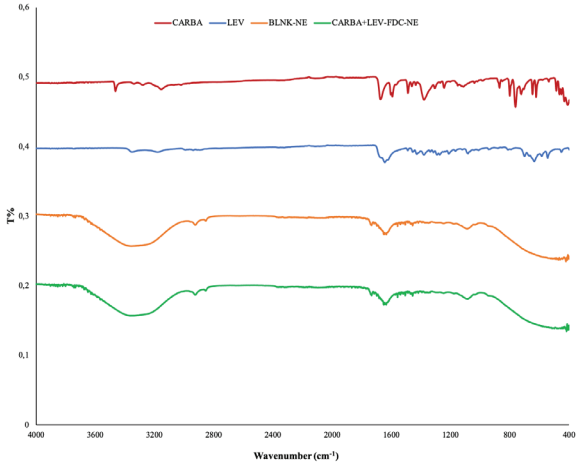

Figure 2. The FT-IR spectra of CARBA, LEV, BLNK-NE and CARBA+LEV-FDC-NE.

Prokhorov et al. (2014) prepared the CARBA-NE formulation and evaluated the anticonvulsant activity of the CARBA-NE against maximum electric shock- or picrotoxin-induced seizures in mice. They reported that the CARBA-NE formulation has more anticonvulsant effect than pure CARBA against seizures.

Echeverri et al. (2020) prepared NE formulation containing CARBA using an ultra-high-pressure homogenization method. The DS, PDI, ZP, pH, and viscosity values of freshly prepared NE formulation were 320.90 nm, <0.3, (-)11.29 mV, 5.9, and 2.27 cP, respectively. The *in vitro* release study for NE formulation was performed in phosphate buffer pH 7.4 using a dialysis bag (MWCO 14 kDa). They reported that the release of CARBA from NE formulation was pretty low. In *in vivo* study, they found

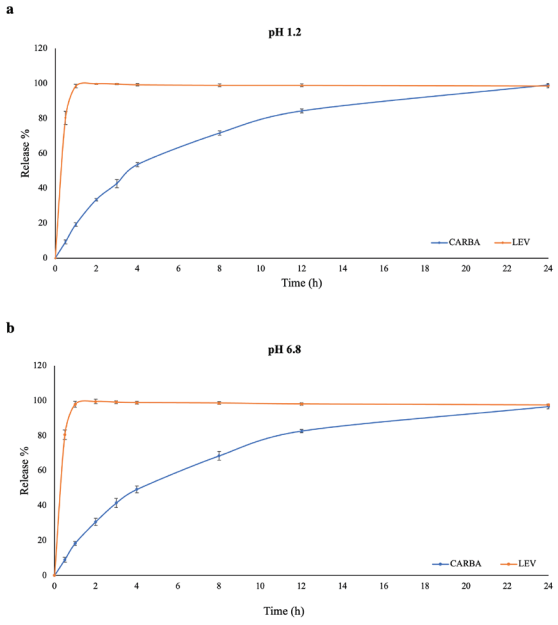

Figure 3. The release profiles of CARBA and LEV from the NE formulation in the different release media (Mean±SD; n=3).

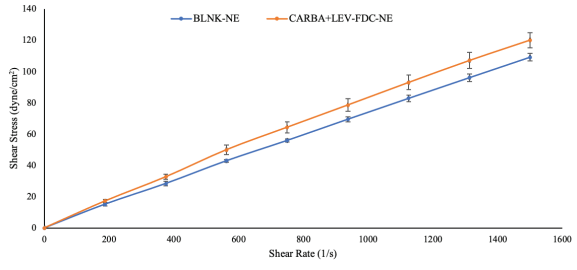

Figure 4. The rheograms of the BLNK-NE and CARBA+LEV-FDC-NE formulations (Mean±SD; n=3).

Table 2. The viscosity values of the BLNK-NE and CARBA+LEV-FDC-NE formulations (Mean±SD; n=3).

Formulation	Viscosity (cP) (at shear rate: 1500 s^{-1})
BLNK-NE	7.28±0.17
CARBA+LEV-FDC-NE	8.01±0.32

SD: Standard deviation; BLNK-NE: Blank nanoemulsion; CARBA+LEV-FDC-NE: CARBA and LEV-containing fixed-dose combination nanoemulsion

that the time to the maximum plasma concentration ($t_{max)}$ for the CARBA-NE formulation or the coarse emulsion containing CARBA administered orally to New Zealand breed rabbits was 45 min and 12 h, respectively.

In another study, an *in vitro* release study for the CARBA-containing NE formulation (The characteristics of this formulation= DS:148.47 nm, PDI: 0.211, ZP: (-)39.68 mV, and CARBA content: 95.25%) was performed in phosphate buffer pH 7.4 using the dialysis bag method (MWCO: 100000 Da). About >90% of CARBA was released from NE formulation within 11 h (Kelmann et al., 2007).

In our study, for oral administration in the treatment of epilepsy, the CARBA+LEV-FDC-NE formulation was successfully prepared. This formulation had suitable *in vitro* characterization results. As a result of the study, it was seen that the droplet size of CARBA+LEV-FDC-NE was 137.56 nm with a narrow size distribution (PDI <0.3), and the ZP of the formulation was sufficient for physical stability. Also, high EE% values were obtained for both active substances. CARBA+LEV-FDC-NE formulation might be beneficial in the treatment of epilepsy.

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