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Evaluation of Brain Targeting Lamotrigine-Loaded Thiolated Chitosan Biodegradable Nanocomposites as Antimigraine vs. Sumatriptan

I M Higazy[1*], H A Zaki[2]
[1] Department of Pharmaceutical Technology, Pharmaceutical and Drug Industries Research Institute, National Research Centre (NRC), Egypt
[2] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Abstract
Thiolated chitosan (TC) has long been perceived as a smart temperature-responsive intranasal polymer. Mucoadhesive properties of thiolated chitosan nanocomposites would allow its binding to nasal mucosa and act as molecular drug-diffusion barrier. Lamotrigine (LTG) is a first-line anti-epileptic dug that has been recommended in migraine prophylaxis, through a number of mechanisms involving GABAergic and glutamatergic receptors, as well as ion channels. Sumatriptan (STP) is a 5-HT agonist that has long been used as anti-migraine, however; its vasoconstrictive effect has limited its use for long time and for certain patients, inspite of its efficiency. In the present study; on one hand, efficiency of thiolated chitosan nanocomposites (TCNC) is examined through a comparative study with orally marketed tablets of both LTG and STP. On the other hand, bioefficiency of LTG as anti-migraine is evaluated in a comparative study with STP. Our target was achieved through physicochemical characterization of drug loaded TCNC, *in-vitro* release and *ex-vivo* permeation studies. Results indicated that the formula LTG2, STP2 and F2 were the optimized, stable formulations with "drug: TC" ratio of "1: 5". Such formulations were then pharmacologically evaluated through *in-vivo* assays involving VonFrey and Hot Plate tests. Results indicated that all intranasal TCNC formulations were significantly effective compared to the oral marketed tablets. LTG2 formulation proved to be the most effective compared to all other formulations. Thus, recommending the possibility of using LTG not only as a prophylactic drug but rather an anti-migraine.

Keywords: Lamotrigine, Thiolated Chitosan, Nanocomposites, Intranasal, Migraine, Triptan

Introduction

Migraine is a common disabling neurological disorder, affecting approximately 14% of the general population. Clinically, it manifests as unilateral, pulsating, severe headache attacks, frequently associated with nausea, vomiting, photophobia and phonophobia [1]. In many cases, migraine is preceded by or associated with transient, reversible, focal neurological symptoms called aura [2]. Minor changes of neuronal excitability at cortical level, have been associated with aura pathophysiology, promoting cortical spreading depression (CSD), involving functional alterations of voltage-gated ionic channels and neuronal glutamate release [3]. Genetically; glutamatergic and GABAergic receptors, namely; unbalanced inhibition-excitation system in the brain, have been linked to migraine pathogenesis. Thereby; GABA and glutamic acid are considered valid biochemical markers of neuronal hyperexcitability governing the cause of migraine aura, and key targets for prophylaxis and treatment of migraine [4].

A number of pathophysiological mechanisms have been commonly observed in both epilepsy and migraine, that some studies suggested that epilepsy is a comorbid condition of migraine. These include disturbed function of voltage-gated sodium and calcium channels and imbalance between GABA-mediated inhibition and excitatory glutamate-mediated transmission. Thereby; antiepileptic drugs have been suggested, and clinically studied, for migraine prevention. However; only few anti-epileptic drugs have proved effective in migraine prophylaxis [5].

Lamotrigine (LTG) is a broad spectrum antiepileptic drug, with low toxicity profile and good patient tolerance. Its mechanism of action involves sodium channel blocking, inducing an indirect inhibition of neuronal glutamate release, hence; blocking cerebral CSD propagation [6]. Clinically, LTG has been previously reported to reduce both frequency and duration of aura n migraine patients [7]. Moreover; LTG has been reported to attenuate neuronal excitability through an indirect effect of on GABA and its ability to depress GABA synaptic inhibition [8]. Furthermore; therapeutic effect of LTG on migraine has been reported in a number of previous studies, when used to treat migraine in epileptic patients [9]. However; its efficiency was also reported in patients with primary recurrent migraine (migraine with aura), chronic migraine, and menstrually-related migraine [10]. Hereby; LTG has long been recommended as preventive treatment of migraine, however; in the present study, we are suggesting its use as a second-line treatment, especially that LTG has good patient compliance, and acceptable safety margin, with low side effect profile, for its long-term administration [11].

Sumatriptan (STP) is a selective 5HT1D/1B agonist, alleviating migraine pain through direct inhibition of trigeminal nerve function, thus; blocking the release of neurotransmitters triggering migraine attacks. This makes STP highly effective in the relief and treatment of sensitive vascular headaches, including migraines with or without aura, as well as cluster headaches [12]. Inspite of its efficiency, triptans have been of limited use especially for patients with vascular disorders, due to their vasoconstrictive effect attributed to the affinity of sumatriptan for 5HT1D receptor which limits the release of endothelium-derived vasodilators and neurotransmitters, maintaining homeostasis of cerebral, coronary, and systemic arterial blood flow [13].

Chitosan is a natural biodegradable polymer that has long been known for its biological applications. In addition, it can be used in designing temperature responsive systems, especially for nasal drug delivery, due to its physiological pH and temperature [14]. Thiolated chitosan (TC) is a polymer formed by amide linkage between amino group of chitosan and carboxylic group of thioglycolic acid, which has superior mucoadhesive properties over unmodified chitosan [15]. At physiological pH, concentration of H+ ions decreases, converting thiol groups (-SH) present on chitosan to thiolated ions (S−), triggering the formation of intermolecular and intramolecular disulphide bonds [16]. This new excipient was found attractive and highly promising for drug delivery especially for intranasal formulations.

Current available treatments for migraine have been associated with poor compliance, low bioavailability, and a number of dose-related side effects including, but not limited to, dizziness, drowsiness, sedation, chest pressure, paresthesia, and dry mouth. Thus; introducing new lines of treatment and prophylaxis as well as proper route of administration and reliable drug delivery system is a must. In the present study, a mucoadhesive thiolated chitosan nanocomposite formulation is designed for intranasal administration for direct and sustained delivery of the drug to its site of action while bypassing the blood brain barrier. In the present study, LTG is investigated for its treatment ability and not only for its prophylactic effect, by comparing its *in-vitro* kinetic release and permeation profiles and *in-vivo* pharmacodynamic profile to STP and oral marketed standards, so it can be considered as second-line treatment for migraine patients.

Materials and Methods

Low molecular weight chitosan (100,000 g/mol, 75–85% deacetylated, 20–300cP viscosity), thioglycolic acid (TGA), ethyl 3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC), Ethanol HPLC-grade (Sigma Aldrich, USA), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl) (Al-Gomhoureya Chemical Company, Egypt), all other chemicals are of analytical grade.

1. Preparation Thiolated Chitosan Nanocomposites (TCNC)

2.1. Thiolation of Chitosan

In a small beaker, 500mg chitosan were weighed and dissolved in 4ml of 1M HCl. To prepare solution (A), the produced chitosan hydrochloride was then treated with 0.75ml TGA in the presence of adequate amount of 50mM EDC-HCL for 30 min (for activation of amino and carboxylic acid groups of chitosan and TGA, respectively). An adequate volume of 1M NaOH was added to solution (A), dropwisely, to adjust pH at 10, while stirring. The mixture was then incubated for 12h at room temperature while stirring on a magnetic stirrer (ARE Hot Plate Stirrer, VELP Scientifica, Italy). Unbound TGA was removed by dialysis using 5mM HCl and 1% NaCl alternatively for three consecutive days. Finally, the dialyzed solution was frozen and lyophilized at -85°C/0.063 mbar (pressure), and stored in air-tight containers at 5°C until further use [17].

2.2. Drug-Thiolated Chitosan Composite

Both lamotrigine and sumatriptan will be loaded into TCNC in "1:3, 1:5 and 1:10" ratios. About 25mg drug (LTG or STP or both combined in 1:1 ratio) were dispersed in 10-15ml of the proper solvent (ethanol: acetone solution 1:3). Once fully dissolved, 0.5g of EDC-HCL was added and mixed for 1h using a high-speed homogenizer (solution A) (Fisherbrand™ 850 Homogenizer, Fisher scientific, USA). Separately, 1g of the previously prepared TCNC was dissolved in 25 ml of 1M HCl with help of a magnetic stirrer (solution B). Solution B was then added to solution A with continuous stirring for 1–2 h at high speed using a homogenizer. To this resultant mixture, 3ml of 5% w/v NaOH solution was added to adjust the pH at 7-7.5. The mixture was then incubated overnight at room temperature while stirring. The resulting dispersion was then filtered then lyophilized under same conditions, previously stated.

2.3. Preparation of Thiolated Chitosan nanoparticles

In a beaker, 250mg of drug loaded thiolated chitosan composite was dissolved in 25ml of 0.5%v/v glacial acetic acid, using a magnetic stirrer (solution A). Then, 100ml phosphate buffer solution with pH adjusted at 10, were prepared. The pH was adjusted using 5%w/v NaOH that was added dropwisely. Solution A was then added dropwisely into the buffer solution, while stirring on high-speed homogenizer for 4h. The resultant solution was cooled then centrifuged at -4ºC for 30 min at 20,000 rpm using Eppendorf® high speed cooling centrifuge (Centrifuge 5920 R - Large Centrifuge, eppendorf, Johannesburg, South Africa, LTD). The residue was then washed with phosphate buffer for three times, then the washing solution was removed and the residue was redispersed into deionized water to obtain the final product.

2. Particle size (PS), Polydispersity Index (PDI) and Zeta Potential (ZP) [18]

Mean particle size (z-average) and distribution (polydispersity index/PDI) of the formed nanocomposites were analyzed using dynamic light scattering (DLS) technique at a scattering angle of 90° at 25°C. Samples were analyzed using (Zetasizer 3000 HSA, MALVERN, UK). Under the same conditions, mean zeta potential was measured using the Malvern Zetasizer. A 100 µl sample was diluted to 3000 µl using deionized water. Samples were stored for three months at 8°C, then, re-evaluated for PS, PDI and ZP.

3. Drug loading capacity and encapsulation efficiency [19]

A 100ml sample of the prepared nanocomposites was loaded to Eppendorf® high speed centrifuge (Centrifuge 5920 R - Large Centrifuge, eppendorf, Johannesburg, South Africa, LTD), and centrifuged at 13000 rpm, under cooling (at -4ºC), for 20 min. The supernatant was discarded, while **t**he residue (containing entrapped drug) was freeze-dried, weighed, then dissolved in a proper HPLC-grade organic solvent and analyzed for amount of loaded drug at the adequate λ_{max} using UV-spectrophotometer (Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer; 115 VAC). The following equations were mathematically employed:

$$Encapsulation\ efficiency(\%) = \frac{calculated\ drug\ content\ (mg)}{theoratical\ drug\ content\ (mg)} \times 100$$

$$Drug\ loading(\%) = \frac{weight\ of\ drug\ (mg)}{weight\ of\ nanoparticles\ (mg)} \times 100$$

4. *In-vitro* release of drug from Thiolated Chitosane Nanocomposites

Dialysis technique using cellulose membrane (Mwt Cut-off 12 kDa, Sigma-Aldrich, USA) was employed. A cellulose bag was filled with 2 ml of drug loaded preparation. Both ends were then closed with suitable clips, then immersed into 100ml of Phosphate buffer saline (release medium), adjusted at pH 6.5 and 35 ± 1 °C, under continuous stirring at 100 rpm (Dristek-USP Bath dissolution tester modified with small volume dissolution kits, USA) [20]. At prescheduled time points, 1 ml sample was withdrawn from the acceptor medium and replaced with equal volume of freshly prepared PBS solution. Collected aliquots were analyzed spectrophotometrically against time. All experiments were performed in triplicates. Mean cumulative release percent was plotted against time and kinetically analyzed using Kinet-DS® software to identify the best fitting kinetic model.

5. *Ex-vivo* Permeation Studies using Sheep Nasal Mucosa [21]

Nasal tissues of freshly slaughtered sheep were surgically removed (Kindly donated from Faculty of Veterinary medicine, Cairo University). Nasal mucosa was peeled from the cartilage of the nasal septum, then washed and stabilized by storing in phosphate-buffered saline (pH 6.5), under cooling till used. Franz-Diffusion cells were used, where nasal mucosa was placed between the donor and receptor compartments as drug diffusion membrane. A 5ml sample of the prepared formulation was placed at the donor compartment, while the receptor compartment was filled with 15ml of simulated nasal fluid (SNF), prepared as described by (Trenkel and Scherließ, 2021) [22], and kept under continuous stirring on magnetic stirrer at (35 ± 1) °C. At a prescheduled time points for 8h, 1ml aliquots were withdrawn from the receptor compartment and replaced with equal volume of fresh SNF medium. Amount of drug permeated through nasal mucosa was determined using UV spectrophotometer at the proper absorbance, against blank. Mean cumulative percentage of the infiltrated drug was calculated.

6. *In-vivo* Studies

Two experiments were designed to test the pharmacological effect of the selected formulation and compare it to positive and negative controls, as well as Oral Standard marketed drug; of dose equivalent to that loaded in the selected formulation. In each experiment, male albino mice (20-25 gm) were used and divided into four groups each comprising 10 mice. Groups 1 to 7, respectively, were: Negative Control (untreated group), Positive control (drug-free intranasal formulation), intranasal LTG loaded TCNC, intranasal STP loaded TCNC, intranasal (LTG + STP) loaded TCNC, Oral Standard LTG (Lamictal™, GlaxoSmithKline, USA), and Oral Standard Sumatriptan (Imitrex™, GlaxoSmithKline, USA), was administered via a flexible polyethylene tube attached to a microsyringe. Animals were housed and properly fed in cages provided with adequate water source, at room temperature. Care-givers were blinded, as each cage comprised 8 mice representing one experimental group. Experiments were held and mice were housed in animal house of Faculty of Medicine, Cairo University. All experiments were performed on awake mice as per the protocol described by Hanson *et al* [23] and the "Arizona State University Institutional Animal Care and Use Committee approved 2010 and updated 2019. All experimentation techniques aligned with the animal ethics standards approved by Cairo University.

6.1. Assessment of Tactile Allodynia

Von Frey test was employed for assessment of mechanical hypersensitivity using electronic von Frey unit. Mice were intraperitoneally injected with a single dose of 15 mg/kg nitroglycerin (NG), for induction of migraine-like pain. Readings were registered on three time points; before NG administration, 45 min after NG injection (baseline) and at different time points after drug administration: 5, 15, 30 and 45min. For negative control group, SNF was intranasally administered. Before the experiment starts, each rat was habituated for 30 min in an individual test compartment with a wire-mesh bottom, to examine pain sensitivity threshold through using single flexible filament with increasing force against the plantar surface of the hind paw of the mouse. Rat behaviour is recorded according to a pain behaviour rating scale. Before NG administration; mice were subjected to the filament pain induction for 3 times alternately in each hind paw, with intermittent 30s breaks between each trial. Once the mice withdraw its paw, the stimulus automatically turns off and the mechanical pressure that evoked the response was recorded. Same procedure was repeated twice, as indicated earlier; 45 min after NG treatment and 45 min after drug administration. Readings obtained infer pain sensitivity threshold.

6.2. Assessment of Thermal Hyperalgesia

Antihyperalgesic characteristics of intranasal LTG TCNC formulation were assessed, using the hot plate test (experimental protocol previously described in 2.9.1., with a slight modification). The hot plate apparatus surface temperature was set at (55 ± 1)°C. Time till licking hind paws or jumping off the hot surface was determined at three different points; first before receiving NG (baseline latency to pain reaction), then after NG administration, and finally after drug administration. A cut-off time of 60s was established to avoid potential paw tissue damage. Mice failing to show any response within the assigned 60s were discarded from the experiment and assigned a score of 60s.

7. Statistical Analysis

Statistical analysis of data was carried out by student t-test and two-way analysis of variance (ANOVA), using SPSS® software at $p < 0.05$, level of confidence. All kinetic modeling was done using Kinet®DS3 software.

Results and Discussion

1. Particle size, size distribution and Zeta Potential

In general as the amount of thiolated chitosan composite increases, both particle size and zeta potential increase significantly ($p \leq 0.05$), as shown in table (1). For analyzing results, each drug group will be independently compared. For LTG loaded TCNC formulations, PS significantly increases, ($p \leq 0.05$), in the following order: LTG1 < LTG2 < LTG3. The same pattern was noticed in formulations loaded with sumatriptan as follows: STP1 < STP2 < STP3, and similarly, formulations loaded with both LTG and STP as follows; F1 < F2 < F3. Generally, there is no significant difference in the PDI values of all formulations ($p > 0.05$). Drug loaded TCNC in the three drug groups showed an

ideal polydispersity index, with PDI ≤ 1.00, indicating a perfect distribution of particles, which would decrease the possibility of particles aggregation or coalescence of the system. Zeta potential values for all formulations indicated stability of all drug loaded TCNC, with values over 30 mv. Besides its polymeric nature, chitosan is also considered as a cationic surfactant. Theoretically, an increase in the surfactant leads to decrease in particle size, however; in case of TCNC, there are two forces controlling particle size, one related to molecular weight and size of TC (steric) and the other related to the charge carried on TC surface (electrostatic). Electrostatic forces, generated due to positive charges of chitosan and the release of H^+ from thiol groups at physiological pH, as previously explained, lead to the high values of ZP noticed in all particles [24]. However; steric properties increasing with the increase of TC content within the nanocomposites lead to relative increase in PS.

After 3 month storage period under refrigeration, values of PS, PDI and ZP were determined again. Formulations, in all drug groups, prepared with "drug: TCNC" ratio of 1:3 and 1: 10, showed significant change in their particle size, PDI and ZP values ($p \leq 0.05$), indicating signs of physical instability, whereas, formulations prepared with 1: 5 ratio, showed no significant changes in their measured parameters ($p > 0.05$). In detail; significant increase in both mean PS and PDI was observed in formulations LTG1, LTG3, STP1, STP3, F1 and F3. However; same formulations showed significant decrease in their ZP ($p \leq 0.05$). Results reveal that steric hindrance had greater effect over electrostatic forces when particles were stored. This was explained by reduced ZP values in some formulations after storage, which resulted in relative accumulation of particles reflected through increase in PS and PDI of some formulations after 3 months storage period.

2. Drug Loading Capacity (DLC) and Encapsulation Efficiency (EE)

By studying the EE and DLC of different formulations, it can be noticed from results presented in table 2, that EE in different drug groups increases significantly as "drug: TCNC" ratio increases ($p \leq 0.05$), whereas DLC initially increases with increasing "drug: TCNC" ratio to "1: 5", then significantly decreases at "1: 10" ratio, ($p \leq 0.05$). Chitosan is known for its biocompatibility and efficiency in drug loading [25]. However; increasing the amount of TC within the formulation causes steric hindrance which increases the ability of the formulation to encapsulate drug, but controversially decreases its loading ability.

Table 1: Particle size, Polydispersity and Zeta Potential along three month period

Drug Group	Formulation	Mean Particle Size (nm ± SD)		Mean Polydispersity Index		Mean Zeta Potential (mv ± SD)	
		Time zero	3 months	Time zero	3 months	Time zero	3 months
Lamotrigine (LTG)	LTG 1	120.54 ± 5.10	146.28 ± 4.87	0.95 ± 0.03	1.80 ± 0.64	31.55 ± 1.04	25.10 ± 2.02
	LTG 2	169.11 ± 3.87	171.37 ± 2.99	0.97 ± 0.04	1.01 ± 0.05	34.93 ± 1.18	35.28 ± 2.03
	LTG 3	202.65 ± 6.03	249.10 ± 5.16	1.01 ± 0.03	2.06 ± 0.19	35.10 ± 1.93	29.98 ± 1.59
Sumatriptan (STP)	STP 1	141.10 ± 4.19	163.25 ± 3.80	0.89 ± 0.04	1.73 ± 0.15	33.84 ± 2.37	27.79 ± 2.66
	STP 2	188.33 ± 3.08	191.05 ± 3.42	0.91 ± 0.04	0.93 ± 0.03	36.17 ± 3.07	37.88 ± 2.41
	STP 3	225.21 ± 5.75	260.28 ± 6.02	1.04 ± 0.05	2.15 ± 0.16	37.41 ± 3.15	31.10 ± 1.17
LTG/STP	F 1	138.22 ± 4.13	151.60 ± 4.26	0.98 ± 0.02	1.67 ± 0.13	33.59 ± 2.44	28.11 ± 1.35
	F 2	181.63 ± 2.99	184.31 ± 3.17	0.99 ± 0.04	1.02 ± 0.02	34.91 ± 3.79	35.92 ± 2.10
	F 3	227.86 ± 3.61	265.99 ± 4.88	1.10 ± 0.03	2.23 ± 0.18	36.23 ± 2.60	31.25 ± 1.97

Table 2: Encapsulation Efficiency and Loading Capacity of various Formulations

Drug Group	Formulation	Encapsulation Efficiency (% ± SD)	Drug Loading Capacity (% ± SD)
(LTG)	LTG 1	73.18 ± 4.15	80.21 ± 5.13
	LTG 2	91.55 ± 2.03	87.92 ± 4.36
	LTG 3	97.70 ± 2.13	69.48 ± 2.87
(STP)	STP 1	65.23 ± 5.09	82.44 ± 3.64
	STP 2	82.41 ± 4.11	89.53 ± 2.90
	STP 3	92.19 ± 2.57	64.69 ± 4.25
LTG/STP	F 1	70.10 ± 2.84	78.18 ± 3.72
	F 2	89.69 ± 3.62	84.93 ± 3.66
	F 3	96.21 ± 1.52	58.94 ± 2.09

3. *In-vitro* drug release studies

Analyzing results of all the previous experiments, formulations prepared with "drug: TCNC" ratio of "1: 5" were considered the optimized formulation, due to their stability and optimized PS, PDI, ZP, EE and DLC. Thereby; formulations LTG2, STP2, F2 were studied for their *in-vitro* release using dialysis bag technique (fig.1). Kinetic analysis of data represented in table 3, revealed that different drugs (LTG and STP) were released according to Korsmeyer-Peppas kinetic order with varying mean release efficiency (MRE) and mean release time (MRT). In general, kinetic mathematical models explain drug release as a function of time, to provide insights about drug mechanism (s) involved in its release. Korsmeyer-Peppas is a kinetic model known to describe non-Fickian release of drug molecules, thus; is the most fitting model usually describing release of drugs from polymeric matrices [26].

Release of LTG loaded in LTG2 formulation showed the highest MRE (52.9 %) and shortest MRT (3.68 h), compared to other formulations. It can also be noticed that release of LTG from LTG2 was significantly better compared to its release from F2. Same attitude was also noticed for the release of STP from both STP2 and F2. This can be justified by the competition between both drugs on the surface release sites of F2, as drug release process is about migration of drug molecules from its position within

the formulation to its outer surface, then to the medium. Accordingly; studying *in-vitro* release is a prerequisite to drug absorption, as it contributes to both kinetic and quantitative aspects of drug bioavailability [27].

4. *Ex-vivo* Permeation studies using Sheep Nasal Mucosa

The permeation profile of the selected formulations was as well studied and kinetically analyzed, as shown in table 4. Similar to release kinetics, Korsmeyer-Peppas was observed as the best fitting model. Furthermore; both LTG and STP showed better permeation profiles when permeated from LTG2 and STP2, respectively; compared to their permeation from F2. Results aligned with drug release pattern and kinetics, demonstrated in section 3.4. Results of both *in-vitro* release and *ex-vivo* permeation would predict similar drug behaviour when examined *in-vivo.*

Table 3: Kinetic Analysis of Release Data of Different Drugs from Optimized TCNC Formulations "1: 5"

Formulation	MCDR (% ± SD)	Kinetic Order	R^2 value	MRE (%)	MRT (h)
LTG 2	98.89 ± 3.21	Korsmeyer -Peppas	0.9998	52.9	3.68
STP 2	92.11 ± 2.27		0.9990	42.8	4.07
F2/LTG	88.07 ± 2.68		1.000	39.9	4.16
F2/STP	80.90 ± 4.02		0.9996	40.1	4.13

* MCDR: Mean Cumulative Drug Released after 8h, F2 is a formulation loaded with 1: 1 ratio of LTG and STP, so it is analyzed twice; once for its LTG content and the other for its STP content, MRE: Mean Release Efficiency, MRT: Mean Release Time

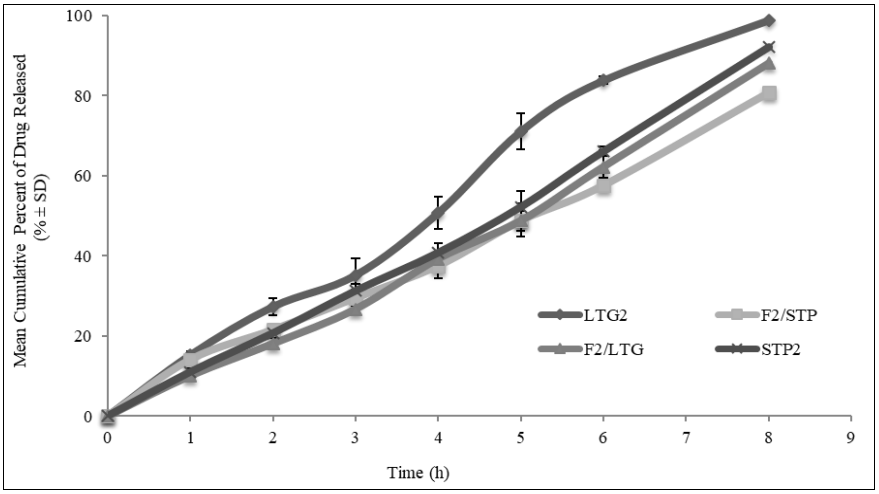

Fig 1: Mean Cumulative Percent of Different Drugs Released from Optimized TCNC Formulations "1: 5"; LTG (lamotrigine), STP (sumatriptan)

Table 4: Kinetic Analysis of Permeation Data of Different Drugs from Optimized TCNC Formulations "1: 5"

Formulation	MCDP (% ± SD)	Kinetic Order	R^2 value	MPE (%)	MPT (h)
LTG 2	91.19 ± 2.88	Korsmeyer -Peppas	0.9998	45.28	4.15
STP 2	86.49 ± 4.10		0.9995	38.31	4.58
F2/LTG	79.45 ± 3.64		0.9998	39.85	4.40
F2/STP	71.26 ± 2.19		0.9997	32.53	4.72

* MCDP: Mean Cumulative Drug Permeation after 8h, F2 is a formulation loaded with 1: 1 ratio of LTG and STP, so it is analyzed twice; once for its LTG content and the other for its STP content, MPE: Mean Permeation Efficiency, MPT: Mean Permeation Time

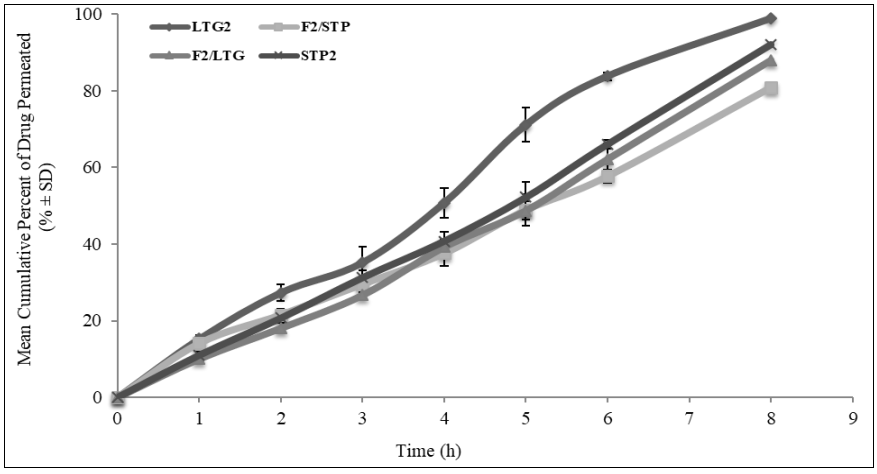

Fig 2: Mean Cumulative Percent of Different Drugs Permeated from Optimized TCNC Formulations "1: 5"; LTG (lamotrigine), STP (sumatriptan)

5. *In-vivo* Studies

5.1. Assessment of Tactile Allodynia

Cutaneous allodynia is one of the symptoms that have been widely associated with chronic migraine attacks, making it a characteristic marker of central sensitization. Considerably; tactile allodynia assay is an evaluation tool of effective treatment response of migraine [28].

Mice in all test groups, before nitroglycerin (NG) injection, showed tolerance to high mechanical pressure applied through Von Frey needles. However; 45min after NG administration, their tolerance level significantly decreased, ($p \leq 0.05$), compared to zero-time; drawing the basal line to our experiment, figure (3). Then, 45min after drug administration [Intranasal LTG-TCNC (LGT2), oral Lamictal™, intranasal STP-TCNC (STP2), oral Sumatriptan™, and intranasal LTG/STP-TCNC (F2)], the pain tolerance of mice in all drug groups, significantly increased ($p \leq 0.05$), compared to the comparative basal line of each group. The insignificant increase, ($p > 0.05$), in pain tolerance noticed in both negative and positive control groups, is mainly attributed to the withdrawal of the NG effect, as both groups received no drug. Different formulations showed efficient pain control in the following order: F2> LGT2> STP2> Sumatriptan™> Lamictal™.

In general, it can be observed that both LGT2 and F2 showed the highest significant pain modulation effect, ($p \leq 0.05$), compared to other formulations, however; there was no significant difference in their comparative effect. More specifically; LTG loaded on TCNC (LTG2) showed significantly higher effect compared to its oral standard tablets of the same dose, indicating the pharmacological efficiency of the prepared intranasal formulation, compared to the oral one known for its expected delayed onset of action. Furthermore; the significantly higher effect of LTG2 compared to STP2, align with a better release profile of LTG compared to STP from TCNC, as previously indicated in sections 3.4 and 3.5. The efficiency of the prepared drug loaded TCNC formulations was also confirmed through the significantly higher effect of STP2 compared to its oral homologue (Sumatriptan™). The TCNC formulations are not only more efficient but rather expected to have a prolonged pharmacological action due to its Chitosan content which provides both prolonged release as well as mucoadhesion.

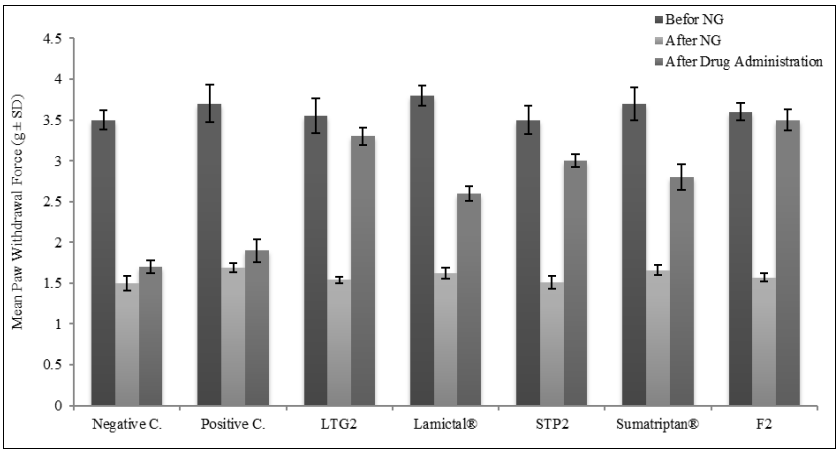

Fig 3: Comparative assessment of Influence of different drugs on Tactile Allodynia through Von Frey Test

Tracking the mice tolerance to the force applied after drug administration at various time points, with zero time considered as the baseline (table 5), the following was observed: at 5min time, only intranasal LTG2, STP2 and F2 showed a significant pain tolerance, compared to their baseline and to their oral homologues. This aligns with the rapid onset of action of intranasal delivery systems compared to the oral ones. The highest pain modulation effect was noticed in LTG2 followed by STP2 then F2, which aligns with the *in-vitro* release and *ex-vivo* permeation profiles of the formulation. Both oral formulations showed no significant difference, $p > 0.05$, compared to their baselines, in terms of their mean paw withdrawal force. The onset of action of oral tablets (Lamictal™ and Sumatriptan™) was observed at 30min time point, with higher effect pronounced in groups receiving Sumatriptan™ compared to those receiving Lamictal™. This might be attributed to the mode of action of both drugs, as the former acts directly on 5-HT receptors giving a direct pain modulation response whereas the latter's action involves a longer pathway to pain modulation.

Table 5: Tracking the effect of drug administration on pain tolerance of mice over various time points through Von Frey Test

Time (min)	Pain Response in Mice Groups Based on Formulation Administered					Mean Paw Withdrawal Response (g ± SD)
	LTG2	Lamictal®	STP2	Sumatriptan®	F2	
0	1.54 ± 0.10	1.62 ± 0.13	1.51 ± 0.12	1.66 ± 0.08	1.57 ± 0.08	
5	2.1 ± 0.05	1.66 ± 0.10	1.8 ± 0.06	1.69 ± 0.09	1.75 ± 0.05	
15	2.8 ± 0.11	1.7 ± 0.13	2.4 ± 0.11	1.8 ± 0.10	2.7 ± 0.12	
30	3.2 ± 0.16	1.9 ± 0.08	2.8 ± 0.13	2.3 ± 0.14	3.1 ± 0.10	
45	3.3 ± 0.14	2.6 ± 0.10	3.0 ± 0.18	2.5 ± 0.13	3.5 ± 0.16	

5.2. Assessment of Thermal Hyperalgesia

Chronic migraine is perceived as a central sensitivity syndrome; identified in patients by widespread pressure hyperalgesia, whose pain sensation is characterized by variation during and between migraine attacks [29]. Thus; thermal hyperalgesia test is used to evaluate migraine

treatments. Results, shown in figure (4), came in accordance with results previously demonstrated through tactile allodynia assay, keeping in consideration that this time pain tolerance was measured through the increase in "mean time of licking response" compared to "mean paw withdrawal force" in von Frey test. After 45min of drug administration: there was no significance in results of both negative and positive controls, as neither has received drugs ($p>0.05$), however; all groups receiving drugs either in its intranasal or oral forms showed significant effect compared to their relative baselines ($p\leq0.05$). Efficiency of different formulations in pain modulation came in the following order: LTG2> STP2> F2> Sumatriptan™> Lamictal™. Worth noting; there was no significance in the effect of both F2 and oral Sumatriptan™, which might be attributed to release kinetics of both LTG and STP from F2 formulation, which is expectedly slowed down due to competition of both drugs on release sites on F2-TCNC surface. However; F2 is expectedly having the advantage of a faster onset compared to oral Sumatriptan™. This was verified by tracking pain modulation over several time points.

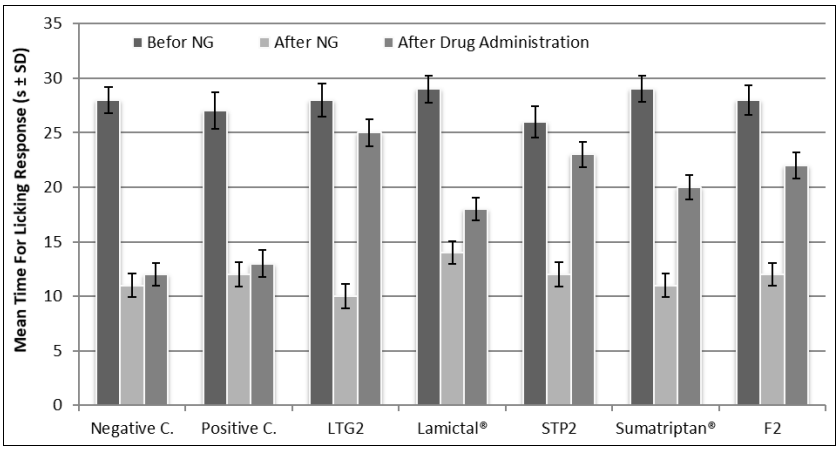

Fig 4: Comparative assessment of Influence of different drugs on Thermal Hyperalgesia employing the Hot Plate Test

The onset of action of both LTG and STP were tracked in different experimental groups after being administered in its different forms, along different time points, table (6). Results came in accordance with results of Von Frey assay, where all intranasal formulations showed an onset of action after 5min of drug administration, compared to 30min for oral marketed drugs.

Table 6: Tracking the effect of drug administration on pain tolerance of mice over various time point through Hot Plate Test

Time (min)	Pain Response in Mice Groups Based on Formulation Administered					Mean Paw Withdrawal Response (g ± SD)
	LTG2	Lamictal®	STP2	Sumatriptan®	F2	
0	10.00 ± 1.03	14.22 ± 1.11	12.00 ± 1.00	11.00 ± 1.15	12.15 ± 1.02	
5	16.50 ± 1.04	14.98 ± 1.75	15.75 ± 1.03	11.29 ± 1.10	15.00 ± 0.86	
15	20.88 ± 0.11	15.10 ± 1.42	17.21 ± 0.11	12.10 ± 1.07	16.77 ± 0.12	
30	23.51 ± 0.16	16.27 ± 1.09	20.48 ± 0.13	17.31 ± 0.14	18.05 ± 0.10	
45	25.11 ± 1.40	18.56 ± 1.39	23.19 ± 1.71	20.44 ± 1.16	22.36 ± 1.20	

Conclusion

Migraine is a brain disorder, affecting a considerable percentage of the population. Its pathophysiology is still questionable and not totally unraveled. Triptans has always been prescribed as first line treatment for migraine patients, however; its vasoconstrictive effect has limited its use, that it became replaced by new classes of anti-migraine drugs as Gepants and Ditans. However; not so many products of the latter alternatives have been approved by the FDA. LTG has been clinically recommended and proved as first line prophylactic drug for migraine, due to its diverse mechanisms of action that involve pain regulators. In the present study, an intranasal LTG loaded TCNC was compared to a STP loaded TCNC and to marketed oral Lamictal™ and Sumatriptan™ formulations and examined via a number of *in-vitro* and *in-vivo* assays. Formulations LGT2, STP2 and F2, proved to me the most stable and optimized formulations, thus; they were further selected for *in-vivo* drug analysis. Formulation LTG2 prepared with "LTG: TCNC" ratio of "1: 5", proved to have optimum physicochemical properties: PS (169.11 ± 3.87 nm), PDI (0.97 ± 0.04), ZP (34.94 ± 1.18 mv), EE (91.55 ± 2.03 %), DLC (87.92 ± 4.36 %), *in-vitro* release (98.89 ± 1.15 %), and *ex-vivo* permeation (91.19 ± 3.05 %). Formulation LTG2 proved to have highly significant efficiency in pain modulation compared to its orally marketed standard, and compared to STP2 and F2 formulations. Therefore; LTG can be recommended not only for prophylaxis of migraine but also for its treatment.

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