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Formulation and Nose-to-Brain Uptake Study of Intranasal Diazepam Nano Emulgel on Rabbits as a Potential Approach to Control Epileptic Emergencies

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Abstract

Drug Delivery to the brain is challenge long has been tried to be tackled, mainly the blood-brain barrier act as a formidable opposing force to drug access. Intranasal administration is one of the methods that aim to overcome this challenge. Diazepam is a CNS-acting positive allosteric modulators of the GABA that is used as anxiolytic, sedative and anticonvulsant. It is a mainstay treatment for epileptic emergencies like status epilepticus. The aim of the study is to formulate Diazepam as a nano emulgel for intranasal administration to provide an alternative route for Diazepam administration to a patient suffering from convulsions using a preliminary animal model based on rabbits. A nano emulgel of Diazepam was tested by preparing a nanoemulsion using Tween 80 as surfactant and ethanol as co-surfactant in 1: 2 ratio (this component is referred to as S_{mix}) with oleic acid as the oil phase with the Ratio of S_{mix} to oil to Water of 50: 10: 40 then converted to an emulgel by addition of 0.5% w/w hydroxy propyl methyl cellulose. Diazepam nano emulgel viscosity was measured and compared to its original nanoemulsion to confirm increase the expected viscosity that's is necessary for long retention in the nasal cavity where mucocilliary clearance is a major issue limiting time available for drug release and its absorption from application site (olfactory region). For the In Vivo study; Six rabbits weighting 1.5-2.25kg were anesthetized and Diazepam nano emulgel was given to them via thin polyvinyl chloride tube into posterior region of their nasal cavities. Cerebrospinal fluid samples were drawn at six time points and analyzed using HPLC and the C_{max}, T_{max} and AUC calculated at a dose of Diazepam of 1.23mg/kg for each rabbit. HPLC results valued the C_{max}, T_{max} and AUC at 1.33mg/ml, 5.2minutes and 14.75 mg/ml.min respectively indicating rapid onset of appearance in CSF at near IV values.

Keywords
Nano emulgel, Intranasal, Diazepam, In Vivo Kinetics, CSF aspiration

For many neurological disorders, satisfactory treatment is almost non-existent due to insufficient entry of therapeutic moieties through blood--brain barrier and brain cerebrospinal fluid barrier to brain [(1)]. Effective brain targeting via overcoming the highly restricted and defiant barriers is a must for successful delivery of such drugs aimed at acting within the central nervous system [(2)].

The available strategies or approaches for drug delivery to CNS fell into either invasive or non-invasive methods [(3)]. Amongst the non-invasive

ones that have been recently focused on for drug delivery to the brain is the intranasal administration of drugs. When administered intranasally, a significant amount of drug can theoretically reach the cerebrospinal fluid and olfactory bulb through olfactory sensory neurons [4] so better targeting is possible to be achieved because of the direct movement of drug from olfactory part or region of the nose into the cerebrospinal fluid compartment of the brain [5].

Many types of nanocarriers have been used in systemic intranasal delivery of drugs like nanomicelles, nanoemulsions, liposomes and nanoparticles as they all confer increasing diffusion through biological membranes, protection against enzymal inactivation and granting blood brain barrier access of non-transportable drugs by masking their physicochemical properties by trafficking them or encapsulating them in these systems [6].

Mouse and rat models are particularly useful for the initial preliminary studies of nose-to-brain absorption of drugs, while rabbit, dog and sheep models are best reserved for pharmacokinetic studies [7]. In humans, the olfactory region covers 10% of the nasal cavity with limited access to inhaled and administered material while In mice and rats the olfactory region covers about 50% of the nasal cavity. Similarities in nasal anatomy of rabbits and dogs existed since they both have branched complex conchae inside their nasal cavitites [8].

Nanoemulsion are thermodynamically stable mixtures of oils, surfactants, and/or co-surfactants mixed at different fixed ratios of these components, with droplet size of the internal phase in the nano range below 100nm [9]. These emulsified liquid mixtures offer a reduction in dose volume and while at the same time opening the opportunity to be designed as spray formulation [10]. The intranasal nanoemulsions are considered as an effective route of administration by allowing direct delivery of the particular dissolved drug to CNS. The O/W nanoemulsions formulation was effectively used to improve the solubility of poorly water-soluble intranasal agents. Several centrally acting drugs for different conditions were delivered as mucoadhesive nanoemulsions by intranasal delivery showed good bioavailability and better cerebral targeting efficiency, indicating the incorporation of mucoadhesive polymers like chitosan in microemulsion components extend the contact time between nasal mucosa, thereby enhancing drug absorption and bioavailability [11].

Epilepsy involves abnormal electrical activity the brain, resulting in either generalized or partial seizures. During a seizure, the goal is to deliver antiepileptic drug to the brain in sufficient amount to decrease the frequency and severity of seizure at minimum side effects [12]. Rapidity of onset of action is the most important criterion for a drug, but also the safety, convenience of administration, and duration of effect are important as well [13].

The current approach to treatment of seizures is producing high levels of antiepileptic agents (Benzodiazepines are first line agents) in the blood, through oral dosage forms or intravenous injections and relatively recently rectal Diazepam was approved in 1997 [14].

All of these methods have a common pitfall, Difficulty of administration during the attack, which the intranasal route can weigh in as a clear winner in this regard. The nasal route has advantages that are not special per se to the nasal route but they can't be achieved by the oral and intravenous route all at the same time, which include non-invasiveness, self-administration, shorter time to onset of effect, higher bioavailability due to avoidance of hepatic first-pass metabolism and, more importantly, bypassing the blood brain barrier with the potential increase in central nervous system availability of the drug [15].

Material and Methods

Preparation of Diazepam Nano Emulgel

Diazepam nanoemulsion was prepared by using Tween 80 as surfactant and ethanol as co-surfactant in 1 : 2 ratio (this component is referred to as S_{mix}) with oleic acid as the oil phase. The Ratio of S_{mix} to oil to Water was 50: 10: 40. Diazepam was accurately weighted and dissolved in a respective amount of oil then the respective quantity of S_{mix} added for oil loaded drug, after that the whole mixture was blended by a vortex mixer for 5 minutes, at the speed of 100rpm. Then the aqueous phase (distilled water) was added gradually to

obtain clear o/w nanoemulsion [16]. The drug was combined with each of these formulations at the dose of 10mg of Diazepam per one milliliter of the respective nanoemulsion.

The original nanoemulsion was completely clear and transparent and was converted into a Nano emulgel by the addition of 0.5% HPMC as a gelling agent that has also mucoadhesive properties

along with certain characteristics that confer it additional advantages for pharmaceutical formulation like preventing crystallization of amorphous drug dispersions during storage and increasing the bioavailability of some poorly soluble drugs [17].

To accurately calculate the amount of HPMC required to achieve the 0.5% w/w, a sample of 80ml of above described nanoemulsion was weighted accurately and was found to have 56gm of weight so the density of the formula is 0.7 gm/ml and 0.28gm of HPMC was added to 80ml of nanoemulsion, stirred with a vortex mixer for few minutes, left for 1 hour then refrigerated overnight [18].

The resultant Nano emulgel was as clear as the original Nano emulsion but with a noticeable increase in viscosity. Higher concentrations of HPMC can also have been used up to 2% but very significant increases in viscosity will render nasal administration more difficult and hinder drug release from its vehicle [19].

Viscosity Measurements of Diazepam Nanogel

The viscosity of the formula before the addition of gelling agent and after the addition were measured. The viscosity and rheological behavior of Diazepam nanoemulsion and nano emulgel were determined with a rotational digital viscometer with R2 spindle [20]. Both samples were measured without dilution with 50ml of each formula was taken and put in a glass beaker and adjusted to the recorded volume level (determined by the mark on the spindle shaft), Measurements started at 37 ±1 °C at six different speeds of 20, 30, 50, 60, 100 and 200 RPM with the corresponding reading recorded at each shear rate. Reverse reading from the 200 RPM to 20 RPM also measured to evaluate any change that may occur during the recovery [21], and the rheological curve was obtained by plotting the recorded viscosity versus the rate spindle rotation.

In Vivo Nose-to-Brain Drug Delivery Study

Study Design

For each run of the study, Six rabbits of mixed local breeds weighting 1.5-2.25kg were purchased from local animal and pet shops and were housed at the animal house of college of veterinary medicine - Tikrit University. All animals were offered free access to food and water, offered reasonable space and kept under regulated room temperature of 25°C and humidity of around 60%. Each rabbit was anesthetized by pre-mixed intramuscular injection of ketamine 100mg/kg and xylazine 10mg/kg [22].

The nasal dose of rabbits was calculated based on the body surface area normalization method and the human equivalent dose (in per kg fashion) of active pharmaceutical ingredients, The Species Factor Km which equals body weight in kilograms divided by body surface area in square meters according to the following equation [23].

$$Animal\ equivalent\ dose\ \left(\frac{mg}{kg}\right) = \frac{Human\ Equivalent\ Dose\ \left(\frac{mg}{kg}\right) * Human\ Km}{Animal\ Km}$$

The recommended dose of Diazepam during epileptic emergencies for adults is 10mg calculated on a 0.4mg/kg basis [24]. The values of Km were 37 and 12 for adult human and rabbit respectively. Dose of Diazepam for rabbits based on BSA normalization method was 1.23 mg/Kg with most rabbits receiving around 2mg dose in total. A polyurethane flexible tube attached to a graduated 1ml syringe were used to accurately push the designated dose into one or two nostrils of the rabbit at a depth of few millimeters [25].

The maximum volume of fluid that can be sprayed or administered to a rabbit is 100-200µl per nostril and careful consideration should be given when administering dose to the rabbit to avoid exceeding the recommended amounts since any additional volume will be sneezed out or swallowed in as a reflex mechanism from the animal to avoid airway occlusion [26]. Since most rabbits in the study required around 2mg dose in total and

the original Diazepam formula was prepared at 10mg/ml concentration, the total volume required was 200µl and it was usually given in one nostril since the animal was more likely to sneeze when the dose was divided into both nostrils even with it being anesthetized. The nasal formulation of drugs was administered using a polyethylene tube attached to a micropipette, inserted into the nostril.

The rabbits were positioned in lateral recumbency with the head flexed 90° to the spine. 6 Cerebrospinal fluid samples were taken at 2, 5, 10, 15 and 30min by forcefully penetrating the cisterna magna (a thin space between cranium and Atlas bone with a 25 mm 22 G needle to the depth of around 1.5cm depending on animal age and weight [27]. CSF samples of 0.5-1 ml were aspired (with sometimes some blood from injection site or internal tissues being sucked along, centrifuged for 5minutes at 400 RPM then the clear supernatant layer was withdrawn into glass tubes and immediately frozen in refrigerator until analysis [28].

Construction of Diazepam Calibration Curve in Cerebrospinal Fluid by HPLC and Sample Measurements.

Diazepam was dissolved in 100% methanol to a concentration of 1 mg⁄mL. From the stock solution, further dilutions were prepared in distilled water to make up calibration curve samples and until development of the calibration curve, The stock solutions were kept at 4° C in a tightly sealed dark vials. The stock solutions were added to blank (control) solution to make up six-point calibration curve with a range 0.0−1.0 µg⁄mL [29].

50µL of CSF samples were injected into a Knauer HPLC system which consist of a pump, a Rheodyne 7125 injector and a UV detector with a thermostatic column compartment. HPLC was performed on a standard analytical C18 column (Knaure;150 Ч4.6 mm; 5µm particle size; 25cm length). The wave length was adjust at the selected λmax 238nm.

The mobile phase for HPLC analysis consisted of acetonitrile, Methanol and distilled water in a ratio of 2:2:1 (v/v) respectively, with no mobile phase additives.

The mobile phase (Acetonitrile: Methanol: Water 2:2:1 v/v) at a flow-rate of 1mL/min was used and the retention time was 1 minute for Diazepam. The auto sampler was maintained at 35° C and the total run time was 10 min [30].

Pharmacokinetic Parameters

Non-compartmental pharmacokinetic profiling for Diazepam cerebrospinal fluid data was done. The maximum cerebrospinal fluid drug concentration (C_{max}) and the time needed to reach maximum cerebrospinal fluid concentration (T_{max}) after nasal administration were directly calculated from the CSF concentration versus time curve. The area under the curve of the CSF drug concentration versus time curve from 0- o.5 h (AUC 0- o.5) and the area under the curve of the CSF drug concentration versus time curve from 0-α (AUC0-α) were calculated for each individual rabbit [31].

Results & Discussions

Viscosity Measurements of Diazepam Nanogel

The viscosity of formulations of Diazepam nanoemulsion and Diazepam Nanogel was measured at various rotational speed (20, 30, 50, 60, 100 and 200 rpm) and at a fixed temperature of 25 °C. Viscosity measurements are shown in the Table 1.

Table 1: Viscosity Measurements of Diazepam Formulations Before and After HPMC Addition

Diazepam Nanogel	Diazepam Nanoemulsion	RPM
860	165	20
595	130	30
430	130	50
370	120	60
212	121	100
144	114	200
213	122	100
378	128	60
434	130	50
N/A	130	30
N/A	N/A	20

The results of viscosity measurements of both formulations were found to be in range of 114 to 165 mPa.sec for Diazepam nanoemulsion and 144 to 860 mPa.sec for Diazepam Nanogel highlighting the significant boost in viscosity gained from HPMC addition to the formula which is known for dramatic hike in viscosity of such formulation upon its addition [32].

It was noticed that both Diazepam

formulations have low viscosities in general sense which is essential to ensure pourability, packing, especially if dedicated for nasal use but the added viscosity of the nanogel form will allow longer retention times, especially with the compound benefit of the mucoadhesive properties of HPMC which is advantageous for longer contact times and consequently better and faster absorption [33]. The results are shown graphically in figure 1 which show the inverse relationship between the rotation speed (shear rate) with the viscosity reduced [34], which indicated that the preparation was flowing in a pseudoplastic manner (shear thinning liquids) [35].

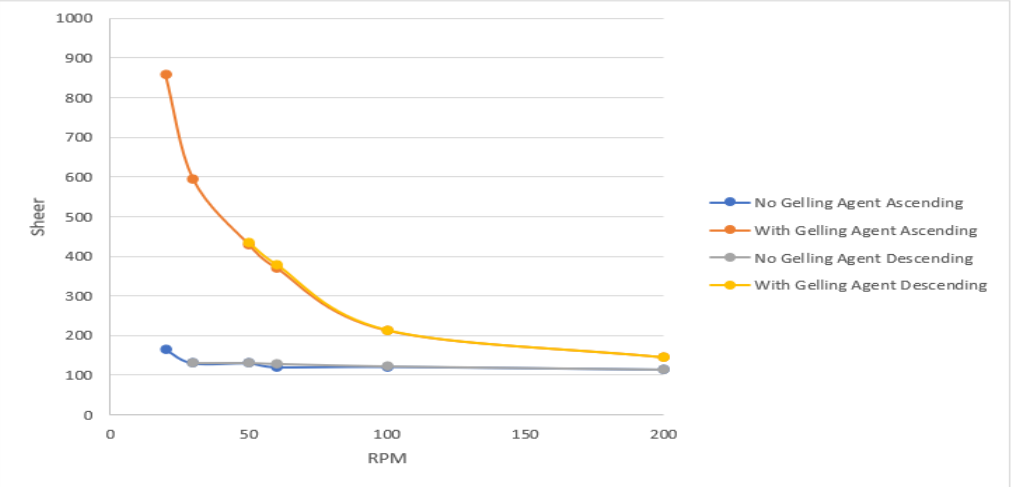
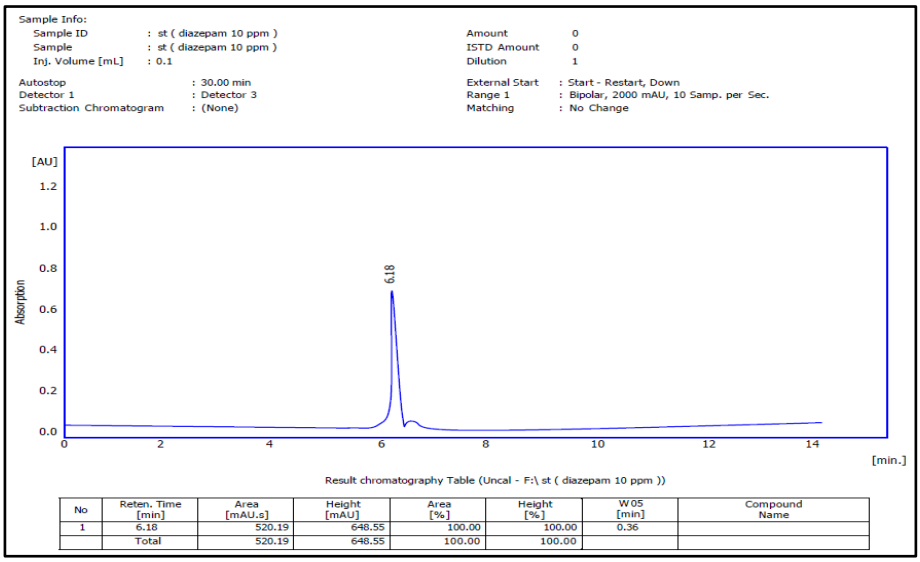


Figure 1: Viscosity values of formula 3 with/without gelling agent at different sheer stress

In-Vivo Nasal Brain Study

The concentration of Diazepam in rabbit CSF had been determined using sensitive and rapid HPLC method that was used to establish quite a reliable calibration curve for the test animals under study. Figure 2 shows retention peaks in Diazepam chromatograms for predetermined amounts of Diazepam (100-5000 ng/mL) in a CSF sample under the chromatographic conditions described earlier.



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	6.18	520.19	648.55	100.00	100.00	0.36	
	Total	520.19	648.55	100.00	100.00		

Figure 2: Chromatogram of diazepam measured in CSF.

The retention time of Diazepam were detected in ethanol and in CSF samples and were recorded to be 6.2 ± 0.5 min.

The calibration curve of Diazepam in CSF showed in figure 3. A straight line with high regression coefficient (r^2 = 0.9991) was obtained by plotting the area under the peak versus the concentration proving linearity of Diazepam's with Beer's law within the concentration range used as shown in figure 4.

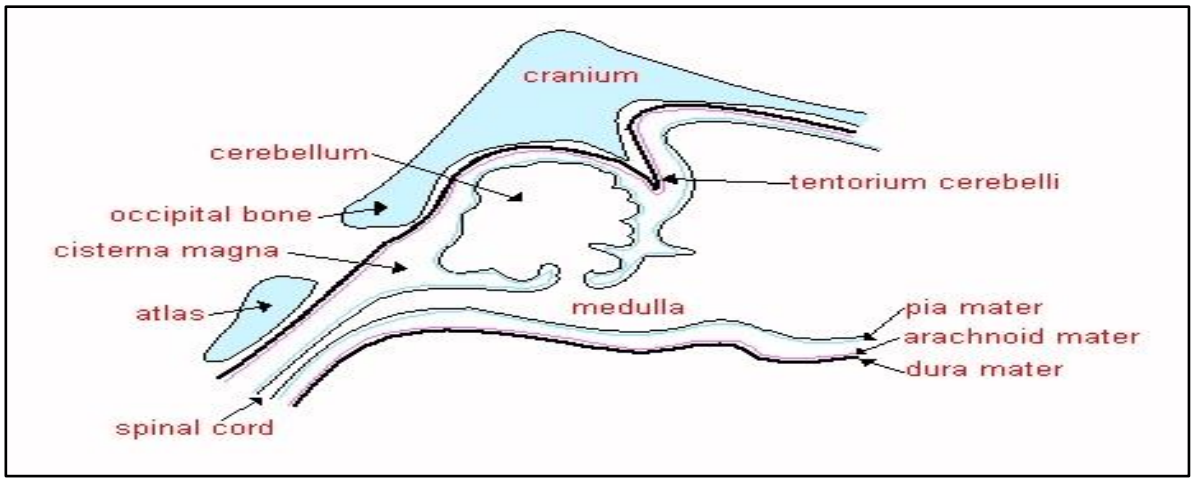


Figure 3: Rabbit's Cisterna Magna Location

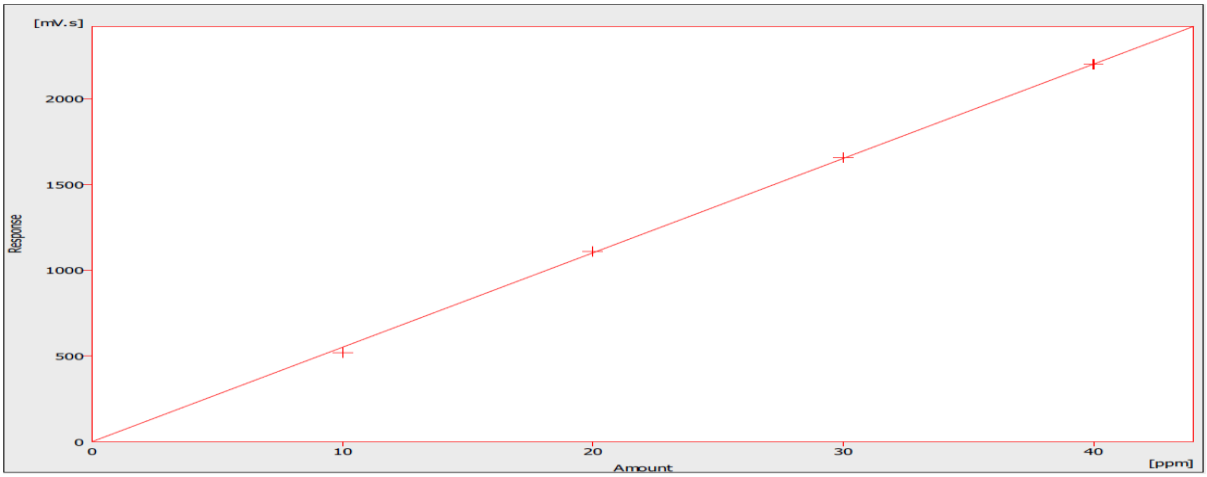


Figure 4: Calibration curve of diazepam in CSF using HPLC

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diazepam - Signal 1 - 6.1 min.

Compound Type	: Ordnr
Left Window	: 0.2 min
Right Window	: 0.2 min
Response Base	: Area
Curve Fit Type	: Linear
Origin	: Curve passes through Origin
Weighting Method	: None
Equation	: Y = 55.05333*X
Correlation Factor	: 0.9998185
Residuum	: 14.40463 [mV.s]
Linearisation X	: None
Linearisation Y	: None

	Response	Amount	Rec No.	Used
1	520.0000	10.0000	1	☒
2	1109.0000	20.0000	1	☒
3	1658.0000	30.0000	1	☒
4	2201.0000	40.0000	1	☒
5	0.0000	0.0000	0	☒
6	0.0000	0.0000	0	☒
7	0.0000	0.0000	0	☒
8	0.0000	0.0000	0	☒
9	0.0000	0.0000	0	☒
10	0.0000	0.0000	0	☒
11	0.0000	0.0000	0	☒
12	0.0000	0.0000	0	☒

Figure 4: Calibration curve-related values of diazepam in CSF chromatogram

The readings were obtained from measuring the Diazepam CSF concentration in 6 rabbits with weights between 1.5-2.25kg requiring the administration of around 1.9-2.8mg of Diazepam per animal and are presented in Table 2 and the resulting average CSF concentration of Diazepam versus time curve is shown in figure 5.

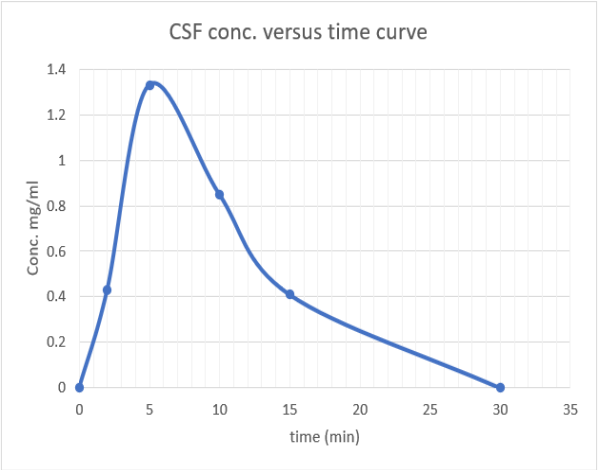

Figure 5: Mean CSF concentrations of diazepam (n=6) versus time in minutes

Table 2: Average CSF Concentration of Diazepam at Different Time Points During In Vivo Study on 6 Rabbits

Time	conc (mg/ml)
0	0
2	0.43
5	1.33
10	0.85
15	0.41
30	undetectable

Results from the table above and with implementation of the trapezoidal method for AUC determination gave the pharmacokinetic data in table 3. Showing reaching peak CSF concentration in 5 minutes which is quite promising for a drug administered intranasally for an emergency. These numbers can be only rivaled by intravenous administration of Diazepam for managing epileptic emergencies. These findings are confirming what previous researchers found when studying potential of intranasal route for Diazepam delivery with results putting intranasal administration head-to-head with intravenous administration of Diazepam for such cases. Such findings were observed in rats [36], rabbits [25] and Dogs [29].

Table 3: Values of Some Pharmacokinetic Parameters Obtained During In Vivo Study

Pharmacokinetic Parameter	Estimated value
C_{max} (mg/ml)	1.33mg/ml
T_{max} (minutes)	5.2 minutes
AUC (mg/ml.min)	14.75 mg/ml.min

Conclusion & Recommendations

The current study results indicated the possibility of administration through nasal route in the form of a nano emulgel that both offers high solubility of the water insoluble Diazepam and the long retention time achieved from the higher viscosity and mucoadhesive feature brought by incorporation of hydroxy propyl methyl cellulose at 0.5% percent in an oleic acid based nanoemulsion of the drug. This could be of tremendous value in acute management of epileptic attacks in which patients usually can't establish IV lines during an attack at home or by non-medically trained caregivers. The intranasal formulation offered rapid onset of action compareable to IV administration and accurate dose calculation for this route require clinical determination of the dose equivalent to the Iv dose in terms of CSF concentration of the drug.

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Conflict of Interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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