Intranasal Mucoadhesive Microemulsions of Clonazepam: Preliminary Studies on Brain Targeting

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ABSTRACT: The aim of this investigation was to prepare clonazepam microemulsions (CME) for rapid drug delivery to the brain to treat acute status epileptic patients and to characterize and evaluate the performance of CME *in vitro* and *in vivo* in rats. The CME were prepared by the titration method and were characterized for globule size and size distribution, zeta potential, and drug content. CME was radiolabeled with ^{99m}Tc (technetium) and biodistribution of drug in the brain was studied in Swiss albino rats after intranasal and intravenous administrations. Brain scintigraphy imaging in rabbits was also performed to ascertain the uptake of the drug into the brain. Pre and postCME formulation treated human nasal mucosa was subjected to transmission electron microscopy to investigate the mechanism of drug uptake across the nasal mucosa. CME were transparent and stable with mean globule size of 15 ± 10 nm and zeta potential of -30 mV to -40 mV. ^{99m}Tc-labeled clonazepam solution (^{99m}Tc CS)/ clonazepam microemulsion (CME)/clonazepam mucoadhesive microemulsion (CMME) were found to be stable and suitable for *in vivo* studies. Brain/blood uptake ratios at 0.50 hour (h) following intranasal CMME, CME, clonazepam solution (CS), and intravenous CME administrations were found to be 0.67, 0.50, 0.48, and 0.13, respectively indicating more effective targeting with intranasal administration and best targeting of the brain with intranasal CMME. Brain/blood ratio at all sampling points up to 8 h following intranasal administration of CMME compared to intravenous was found to be twofold higher indicating larger extent of distribution of the drug in brain. Rabbit brain scintigraphy also showed higher intranasal uptake of the drug into the brain. Transmission electron microscopy revealed significant accretion of CMME within interstitial spaces and paracellular mode of transport due to stretching of the tight junctions present in the nasal mucosa. This investigation demonstrates a more rapid and larger extent of transport of clonazepam into the rat brain with intranasal CMME, which may prove useful in treating acute status epileptics.

Keywords: intranasal; microemulsion; clonazepam; radiolabel; TEM; brain targeting; mucoadhesion; biodistribution

INTRODUCTION

Status epileptics is a neurological disorder, which requires quick management of seizures in order to avoid the risk of permanent brain damage. Clonazepam, a benzodiazepine derivative is used

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widely in the treatment of status epileptics. Clonazepam is preferred over other benzodiazepines due to its longer duration of action (24 h).[1] Clonazepam, the drug of choice in suppression of myoclonic seizures, acts by increasing the effectiveness of the inhibitory neurotransmittor, gamma amino butyric acid, within the central nervous system.

Presently, clonazepam is available in tablet and injectable dosage forms (Revotril, Hoffmann-La-Roche, NJ, USA http://www.fda.gov/cder/foi/nda/index.htm).[2] These formulations release clonazepam into the peripheral circulation resulting in limited drug uptake across the blood–brain-barrier and in drug distribution to nontargeted sites.[3] Although intravenous administration provides rapid seizure suppression, an alternative route of drug delivery is needed since oral and intravenous routes for delivering drugs are sometimes impractical and/or inconvenient,[1] for instance, because of a delay in hospitalization of the patient, lack of an available hospital facility or a patient condition incompatable with oral ingestion of a tablet dosage form.[4]

Previous studies have demonstrated that intranasal administration offers a practical, noninvasive, and an alternative route of administration for rapid drug delivery to the brain.[5–7] Intranasal drug delivery also offers the advantages that drugs can be administered simply, cost effectively, and conveniently.[8] Direct transport of drugs to the brain circumventing the brain-barriers following intranasal administration provides a unique feature and better option to target drugs to brain.[9–11] However, to enhance effectiveness of the drug, a few issues should be carefully considered by the formulator when designing intranasal drug delivery.[11,12] The formulation should be designed so as to provide rapid transport of drug across nasal mucosa and longer residence time in nasal cavity.[13] Microemulsions, by virtue of their lipophilic nature and having low globule size, are widely explored as a delivery system to enhance uptake across mucosa.[14] Addition of a mucoadhesive agent such as a polyelectrolyte polymer helps in retention of the formulation on the nasal mucosa.[15] Evidences of intranasal drug delivery systems formulated using mucoadhesive agent and its benefits in enhancing nose-to-brain drug transport have been reported by various scientists in the literature.[16,17]

The objective of this investigation was to prepare and optimize rapid brain-targeted CME/CMME and to characterize and evaluate its performance *in vitro* and *in vivo* in rats. Hence, the present study deals with the preparation of CME/CMME, biodistribution, and elucidation of transnasal transport mechanism of the drug to justify its role in the treatment of acute status epileptics. It was hypothesized that microemulsion/mucoadhesive microemulsion based alternative drug delivery systems will result in rapid nose-to-brain transport of clonazepam and greater drug transport and distribution into and within the brain. This can help to maximize the therapeutic index of the drug, reduce side effects, decrease the dose and frequency of dosing, and perhaps even the cost of the therapy.

MATERIALS AND METHODS

Chemicals

Clonazepam was a gift from Torrent Pharmaceuticals (Ahmedabad, India). Propylene glycol was purchased from ISP Technologies (Mumbai, India). Polysorbate 80, ICI chemicals (Mumbai, India) and polyoxyethylene 35-ricinoleate, BASF (Mumbai, India) were received as gifts. Polycarbophil (AA-1, pharmagrade, mol. weight 3.50 billion) was purchased from Noveon (Mumbai, India). Diethylene triamine penta acetic acid (DTPA) and stannous chloride dihydrate ($SnCl_2.2H_2O$) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium pertechnetate, separated from molybdenum-99 (99 m) using a solvent extraction method, was provided by Regional Center for Radiopharmaceutical Division (Northern Region), Board of Radiation and Isotope Technology (BRIT, Delhi, India). All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Preparation and Characterization of Microemulsion

Clonazepam solution (CS) was prepared by addition of Clonazepam (CL, 50 mg) to 8 mL distilled water and ethyl alcohol mixture (90:10 v/v) with stirring. The pH was adjusted to 3.50 ± 0.25 using glacial acetic acid (0.10 mL). The dispersion was stirred for 15 min to obtain clear solution. The final volume was made up to 10 mL using distilled water. CME was prepared (5 mg/mL clonazepam) using medium chain triglyceride as an oil (10% w/w), polyoxyethylene-35-ricinoleate as a surfactant (S, 26.67% w/w), polysorbate 80 as a cosurfactant (CoS, 13.33% w/w), and propylene glycol

(50% w/w) as an anhydrous continuous phase. Formulations were prepared by dissolving CL at 55°C ± 5°C in S, CoS, and oil mixture. The resultant solution was cooled to ambient temperature. Propylene glycol was added gradually with continuous stirring, which resulted into transparent and homogenous CME (% transmittance at 630 nm >99%). CMME was prepared by addition of polycarbophil (0.50% w/w) to CME with continuous stirring. The dispersion is stirred for 15 min.

A high-performance liquid chromatography (HPLC) method[18] with detector adjusted at 254 nm wavelength was used for determination of CL. Kromasil C_{18} column (5 μm, 30 × 4.60 mm ID) was used for separation. Mobile phase (water, methanol, and acetonitrile (4:3:3)) was degassed and isocratically run at a flow rate of 1 mL/min and the injection volume was 25 μL.

The globule size determination[19] was performed using photon correlation spectroscopy with in-built Zetasizer (Model: Nano ZS, Malvern Instruments, Worcestershire, UK WR141XZ) at 633 nm. Helium-neon gas laser having intensity of 4 mW was the light source. The equipment was programmed to provide 18 mm laser width. Measured electrophoretic mobility (μm/s) using small volume disposable zeta cell is converted to zeta potential[19] by in-built software based on Helmholtz- Smoluchowski equation.

Radiolabeling of Clonazepam Solution and its Microemulsions

Clonazepam solution (CS), clonazepam microemulsion (CME), and clonazepam mucoadhesive microemulsion (CMME) were radiolabeled using ^{99m}Tc by direct labeling method.[20,21] One milliliter of either CS (5 mg/mL), CME (5 mg/mL), or CMME (5 mg/mL) was taken and stannous chloride dihydrate solution (100 μg in 100 μL of 0.10 N HCl) was added. The pH was adjusted to 7.00 ± 0.20 using 50 mM sodium bicarbonate solution. To the resultant mixture, 1 mL of sterile ^{99m}Tc-pertechnetate (75 to 400 MBq) was added gradually over a period of 60 s with continuous mixing. The mixture was incubated at room temperature for 30 min with continuous nitrogen purging. The final volume was made up to 2.50 mL using 0.90% (w/v) sodium chloride (normal saline) solution.[22]

The radiochemical purity[23] of ^{99m}Tc-CS (Technetium-99m labeled CS), ^{99m}Tc-CME (Technetium-99m labeled CME), and ^{99m}Tc-CMME (Technetium-99m labeled CMME) were assessed using ascending instant thin layer chromatography. Silica gel coated fiber glass sheets (Gelman Sciences, Inc., Ann Arbor, MI) and dual solvent systems consisting of acetone and pyridine: acetic acid: water (3: 5: 1.5 v/v) were used as mobile phases. The effect of incubation time, pH, and stannous chloride concentration on radiolabeling efficiency were studied to achieve optimum reaction conditions.[22,23] The radiolabeled formulations were challenged for bonding strength using diethylene triamine penta acetic acid (DTPA).[24] The optimized radiolabeled formulations were assessed for *in vitro* stability in normal saline solution and in rat plasma.[25] The composition, drug content, globule size, zeta potential, and radiolabeling efficiency for clonazepam formulations are recorded in Table 1. Consequently, the optimized stable radiolabeled formulations were used to study bio-distribution in rats.

Bio-Distribution Studies

All experiments conducted on animals were approved by the Social Justice and Empowerment Committee for the purpose of control and supervision on animals and experiments, Ministry of Government of India. Swiss albino rats (male, aged 4–5 months), weighing between 200 and 250 g were selected for the study.

Four rats for each formulation per time point were used in the study. The radiolabeled complex of ^{99m}Tc-CME (100 μCi/100 μL) containing 0.033–0.041 mg clonazepam (equivalent to 0.16–0.20 mg/kg body weight (B.W.)) was injected through tail vein of Swiss albino rats. Similarly, radiolabeled complex of ^{99m}Tc-CS/CME/CMME (100 μCi/20 μL) containing 0.033–0.041 mg clonazepam (equivalent to 0.16–0.20 mg/kg B.W.) was administered (10 μL) in each nostril. Prior to nasal administration of the formulations, the rats were anaesthetized using 50 mg/kg ketamine intramuscular injection and the formulations were instilled into the nostrils with the help of micropipette (100 μL) attached with low density polyethylene tube having 0.1 mm internal diameter at the delivery site. The rats were held from the back in slanted position during nasal administration of the formulations. The rats were sacrificed with mercy at predetermined time intervals and blood was collected using cardiac puncture. Subsequently, different tissues/organs including brain and spinal cord were dissected, washed twice using normal saline solution, and made free from adhering

Table 1. Composition and Characterization* of Clonazepam Formulations

Abbreviation	Formulation	O (%)	S (%)	CoS (%)	AQ (%)	Drug Content (%)	Globule Size (nm)	Zeta Potential (mV)	Radiolabeled Complex (%)
CS	Clonazepam solution	—	—	—	100%	99.67 ± 0.18	—	—	96.35 ± 0.62
CME	Clonazepam microemulsion	10	26.67	13.33	50.0	99.20 ± 0.14	15.21 ± 2.42	−29.88 ± 1.45	96.21 ± 0.31
CMME	Clonazepam mucoadhesive microemulsion	10	26.67	13.33	50.0	98.90 ± 0.39	11.27 ± 1.80	−39.10 ± 0.92	96.17 ± 0.12

*The results are mean values ± SEM derived from six different experimental batches. O is denoted for Oil Phase (Medium chain triglyceride), S for surfactant (polyoxyethylene-35-ricinoleate), CoS for cosurfactant (polysorbate 80), and AQ is denoted for aqueous phase (propylene glycol). The formulations (CS, CME, and CMME) mentioned in Table 1 contains clonazepam 5 mg/mL.

tissue/fluid and weighed. The radioactivity present in each tissue/organ was measured using shielded well-type gamma scintillation counter. The radiopharmaceutical uptake per gram in each tissue/organ was calculated as a fraction of administered dose.[22] The results of radioactivity in different organs are recorded in Table 2. The pharmacokinetic parameters were derived from Table 2 and Figure 1A and B using Kinetica (version 4.10, Innaphase, Philadelphia, PA, USA) and recorded in Table 3. To evaluate the brain targeting efficiency, two indexes (DTE (%) and DTP (%)) were adopted as mentioned below.[26,27]

Drug targeting efficiency (DTE (%))[26,27]: DTE (%) represents time average partitioning ratio.

$$\mathrm{DTE}\,(\%) = [\{(\mathrm{AUC_{brain}}/\mathrm{AUC_{blood}})\}_{\mathrm{i.n.}} / \{(\mathrm{AUC_{brain}}/\mathrm{AUC_{blood}})\}_{\mathrm{i.v.}}] * 100 \qquad (1)$$

In order to define nose-brain direct transport clearly, "the brain drug direct transport percentage (DTP (%))"; which has been derived from equations (2) and (3).

$$\mathrm{DTP}\ \% = \{(\mathrm{B_{i.n.}} - \mathrm{B_x})/\mathrm{B_{i.n.}}\} * 100 \qquad (2)$$

$$\text{Where, } B_{\mathrm{x}} = (\mathrm{B_{i.v.}}/\mathrm{P_{i.v.}}) * (\mathrm{P_{i.n.}}) \qquad (3)$$

$\mathrm{B_x}$ = Brain AUC fraction contributed by systemic circulation through the blood–brain barrier (BBB) following intranasal administration.

$\mathrm{B_{i.v.}} = \mathrm{AUC}_{0\rightarrow 480}$ (brain) following intravenous administration.

$\mathrm{P_{i.v.}} = \mathrm{AUC}_{0\rightarrow 480}$ (blood) following intravenous administration.

$\mathrm{B_{i.n.}} = \mathrm{AUC}_{0\rightarrow 480}$ (brain) following intranasal administration.

$\mathrm{P_{i.n.}} = \mathrm{AUC}_{0\rightarrow 480}$ (blood) following intranasal administration.

AUC = Area under the curve.

Reports in the literature reveal that the drug uptake into the brain from the nasal mucosa mainly occurs via three different pathways.[11,28] One is the systemic pathway by which some of the drug is absorbed into the systemic circulation and subsequently reaches the brain by crossing BBB. The others are the olfactory pathway and the trigeminal neural pathway by which partly the drug travels directly from the nasal cavity to CSF and brain tissue.[10,28] We can conclude that the amount of drug reaches in the brain tissue after nasal administration is attributed to these three pathways. Since, clonazepam displays linear pharmacokinetics,[29] the amount of drug is proportional to AUC. Thus, we can assume that the brain

Table 2. Compartmental Distribution of ^{99m}Tc-CME (i.v.), ^{99m}Tc-CME (i.n.), ^{99m}Tc-CMME (i.n.), and ^{99m}Tc-CS (i.n.) at Different Time Intervals in Normal Swiss Albino Rats*

Formulation and Route of Administration	Distribution of Clonazepam in Blood and Brain Compartments at Different Sampling Time Points					
	Organ/Tissue	0.50 h	1.0 h	2.0 h	4.0 h	8.0 h
CME (i.v.)	Blood	3.81 ± 0.35	2.49 ± 0.56	2.26 ± 0.39	1.44 ± 0.32	1.28 ± 0.29
	Brain	0.48 ± 0.12	0.80 ± 0.09	0.90 ± 0.06	0.66 ± 0.04	0.23 ± 0.01
CMME (i.n.)	Blood	1.58 ± 0.19	1.68 ± 0.46	1.95 ± 0.52	2.50 ± 0.28	1.37 ± 0.37
	Brain	1.06 ± 0.09	1.35 ± 0.22	1.33 ± 0.38	1.24 ± 0.34	0.49 ± 0.08
CME (i.n.)	Blood	1.24 ± 0.08	1.39 ± 0.11	1.55 ± 0.09	2.02 ± 0.05	1.45 ± 0.03
	Brain	0.62 ± 0.04	0.98 ± 0.07	1.03 ± 0.08	0.84 ± 0.06	0.37 ± 0.06
CS (i.n.)	Blood	0.81 ± 0.19	1.06 ± 0.34	2.15 ± 0.48	1.79 ± 0.49	0.94 ± 0.33
	Brain	0.39 ± 0.15	0.48 ± 0.12	0.44 ± 0.18	0.36 ± 0.06	0.33 ± 0.04
CME (i.v.)	Brain/blood	0.126 ± 0.09	0.321 ± 0.11	0.398 ± 0.12	0.458 ± 0.04	0.180 ± 0.11
CMME (i.n.)	Brain/blood	0.671 ± 0.12	0.803 ± 0.05	0.682 ± 0.14	0.496 ± 0.03	0.358 ± 0.16
CME (i.n.)	Brain/blood	0.500 ± 0.04	0.705 ± 0.06	0.665 ± 0.05	0.416 ± 0.08	0.255 ± 0.12
CS (i.n.)	Brain/blood	0.481 ± 0.16	0.453 ± 0.02	0.205 ± 0.07	0.316 ± 0.09	0.351 ± 0.07

*The rats were administered with 100 μCi ^{99m}Tc-clonazepam and the radioactivity was measured in percent per gram of tissue of the administered dose. Each value is the mean ± SEM of four estimations. Radioactivity was measured at 0 h and all the measurements were performed using 0 h sample of corresponding tissue/organ as blank sample.

AUC fraction contributed by systemic circulation through BBB (represented by B_x), divided by plasma AUC from nasal route is equal to that of i.v. route (see Equation (1)). Therefore, DTP (%) represents the percentage of drug directly transported to the brain via the olfactory pathway and the trigeminal neural pathway. DTP (%) and DTE (%) were calculated using tissue/organ distribution data following intranasal and intravenous administrations and are recorded in Table 4.

Gamma Scintigraphy Imaging

The New Zealand rabbits (2.00–2.50 kg) were selected for the study. The radiolabeled complex of ^{99m}Tc-CME (500 μCi/500 μL) containing 0.40–0.50 mg CL (equivalent to 0.16–0.20 mg/kg) was intravenously injected through the ear vein of the rabbit. Similarly, the radiolabeled complex of ^{99m}Tc-CS/CME/CMME (100 μCi/100 μL) containing 0.40–0.50 mg CL (equivalent to 0.16–0.20 mg/kg B.W.) was administered (50 μL in each nostril). The rabbits were held from the back in slanted position during nasal administration of formulations. The rabbits were anaesthetized using 1 mL ketamine hydrochloride intramuscular injection (50 mg/mL) and placed on the imaging platform. Imaging was performed using Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens AG; Erlanger, Germany) gamma camera.[22,30] The scintigraphy images following intravenous and intranasal administrations of CMME are shown in Figure 2.

Transmission Electron Microscopy

Human nasal mucosa was collected after proper informed consent of donor and washed with phosphate buffered saline twice to remove adhered tissues. The nasal mucosa was stored at 2–4°C in a cotton gauze impregnated with normal saline solution till further use. Human nasal mucosa was kept within the CS, CME, and CMME for 12 h to study the formulation uptake across nasal mucosa, mechanism of drug uptake, and toxicity of the formulations on the nasal mucosa cells. Subsequently, formulation treated nasal mucosa was exposed (3 h) to 100 mM phosphate buffer solution (pH 6.5) for removal of formulation and toxicity of formulation on nasal mucosa cells was studied. Nasal mucosae, with/without formulation treatment and after washing, were fixed using 2.50% (v/v) gluteraldehyde solution in water for 3 h at 25 ± 2°C. The fixed nasal mucosae were washed thrice using 100 mM phosphate buffer (pH 6.50). Washed nasal mucosae were postfixed in 1% w/v osmium tetroxide solution for 3 h; fresh osmium tetroxide was replaced every 30 min. The nasal mucosae samples were washed, dehydrated through acetone grades, and infiltered in araldite: dodeceny succinic anhydride

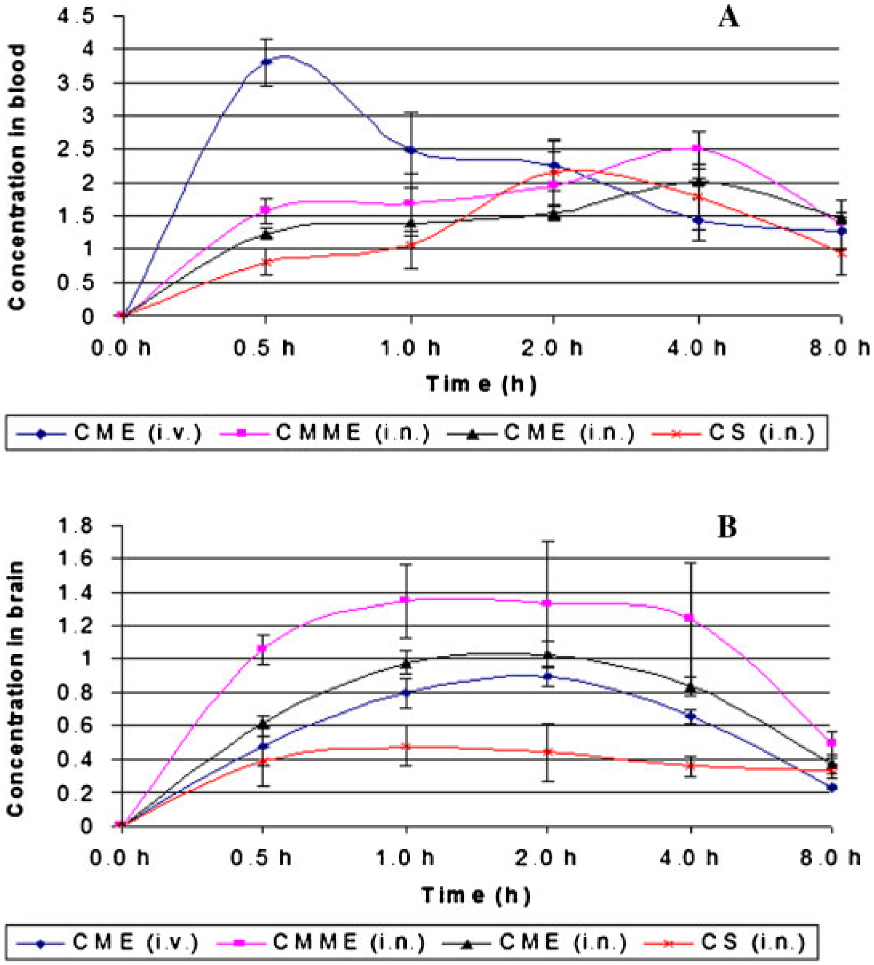

Figure 1. (A) CL concentration in rat blood at different time intervals following CME (i.v.), CMME (i.n.), CME (i.n.), and CS (i.n.) administrations. (B) CL concentration in rat brain at different time intervals following CME (i.v.), CMME (i.n.), CME (i.n.), and CS (i.n.) administrations. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

Table 3. Pharmacokinetics of ^{99m}Tc-CME (i.v.), ^{99m}Tc-CME (i.n.), ^{99m}Tc-CMME (i.n.), and ^{99m}Tc-CS (i.n.) at Different Time Intervals in Normal Swiss Albino Rats*

	Organ/Tissue	C_{max} (%/g)	T_{max} (h)	$AUC_{0\rightarrow 480}$ (h* %/g)	$AUC_{0\rightarrow \infty}$ (h* %/g)	K_{el} (L/h)	$T_{1/2}$ (h)
Intravenous CME	Blood	3.81 ± 0.23	0.50 ± 0.10	26.67 ± 1.12	27.90 ± 1.05	0.14 ± 0.05	5.10 ± 0.50
	Brain	0.90 ± 0.04	2.0 ± 0.10	5.78 ± 0.48	5.82 ± 0.62	0.21 ± 0.03	3.28 ± 0.25
Intranasal CMME	Blood	2.50 ± 0.16	4.0 ± 0.35	26.98 ± 0.26	28.30 ± 0.21	0.13 ± 0.03	5.20 ± 0.15
	Brain	1.35 ± 0.15	1.0 ± 0.10	10.38 ± 0.55	10.47 ± 0.31	0.20 ± 0.05	3.55 ± 0.10
Intranasal CME	Blood	2.02 ± 0.11	4.0 ± 0.25	20.03 ± 0.32	21.18 ± 0.47	0.12 ± 0.02	5.59 ± 0.45
	Brain	1.03 ± 0.07	2.0 ± 0.25	5.45 ± 0.45	5.49 ± 0.34	0.19 ± 0.06	3.29 ± 0.28
Intranasal CS	Blood	2.15 ± 0.23	2.0 ± 0.15	16.42 ± 0.17	16.62 ± 0.18	0.11 ± 0.04	5.54 ± 0.42
	Brain	0.48 ± 0.06	1.0 ± 0.10	4.65 ± 0.24	5.02 ± 0.62	0.19 ± 0.10	3.23 ± 0.23

*The rats were administered with 100 μCi ^{99m}Tc-clonazepam and the radioactivity was measured in percent per gram of tissue of the administered dose. Each value is the mean ± SEM of four estimations.

Table 4. Brain Targeting Efficiency and Direct Nose-to-Brain Transport* Following Intranasal Administration of ^{99m}Tc-CMME, ^{99m}Tc-CME, and ^{99m}Tc-CS

Formulation and Route of Administration	Brain Targeting Efficiency (DTE (%))	Direct Nose to Brain Transport (DTP (%))
Intranasal (CMME)	229 ± 4	44 ± 2
Intranasal (CME)	131 ± 3	20 ± 1
Intranasal (CS)	124 ± 1.5	23 ± 1.5

*Parameters derived using values of four different estimation estimations and each value is mean ± SEM.

mixture (1: 1.32) for 24 h. The resin mixture was removed and nasal mucosa samples were embedded in pure resin, the samples were cured by subjecting at 60 ± 2°C for 72 h. Ultra-thin sections (20–30 μm) were taken using microtome and placed on 200 mesh formwar coated copper grids. The copper grids containing nasal mucosa samples were stained using uranyl acetate and lead citrate (Reynolds, SPI Supplies, West Chester, PA, USA). To study morphological changes of epithelial cells and tight junctions, nasal mucosa samples were scanned using JEOL 100 CX transmission electron microscope (Jeol, Japan) equipped with 20 μm aperture at 80 kVo. Electron micrographs of the normal nasal mucosa, formulation treated nasal mucosa, and washed nasal mucosa are shown in Figure 3A.

Statistical Analysis

All data are reported as mean ± SEM and the difference between the groups were tested using Student's *t*-test at the level of $p < 0.05$. More than two groups were compared using ANOVA and differences greater at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

CS, CME, and CMME were prepared and characterized for assay, globule size, and zeta potential. The CL content was found to be 99.67%, 99.20%, and 98.90% for CS, CME, and CMME, respectively. The mean globule size and zeta potential of CME were found to be 15.21 nm and −29.88 mV and for CMME were found to be 11.27 nm and −39.10 mV, respectively. The CME showed net negative charge and addition of mucoadhesive agent further contributed negatively to the system. This may be attributed to the fact that increase in surfactant level resulted in a decrease in surface tension and surface free energy of the formed micelles. Therefore, net negative charge (anionic) of the microemulsion increased.[31] The prepared microemulsions were expected to have good physical stability with respect to phase separation and/or flocculation as zeta potential[32,33] is less than −30 mV.

CS, CME, and CMME formulations were effectively radiolabeled with Technetium-99m (^{99m}Tc), optimized for maximum labeling efficiency and stability. Radiochemical purity achieved was 96.35%, 96.21%, and 96.17% for CS, CME, and

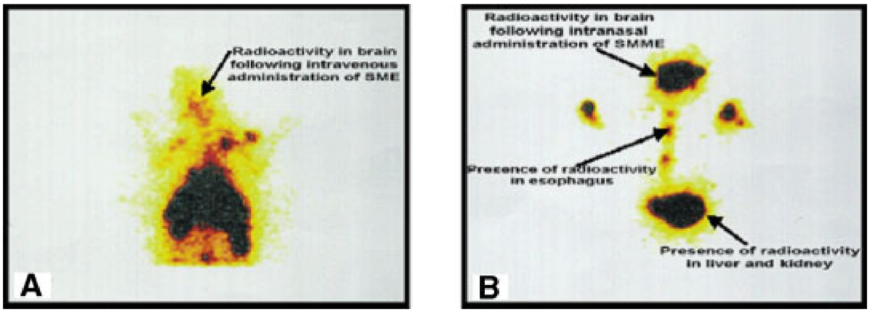

Figure 2. (Left) Gamma scintigraphy of anteroposterior (AP) view of rabbit following intravenous administration of CME. (Right) Gamma scintigraphy (AP view) of rabbit following intranasal administration. Rabbits were administered 1mCi radioactivity by intravenous and intranasal administration. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

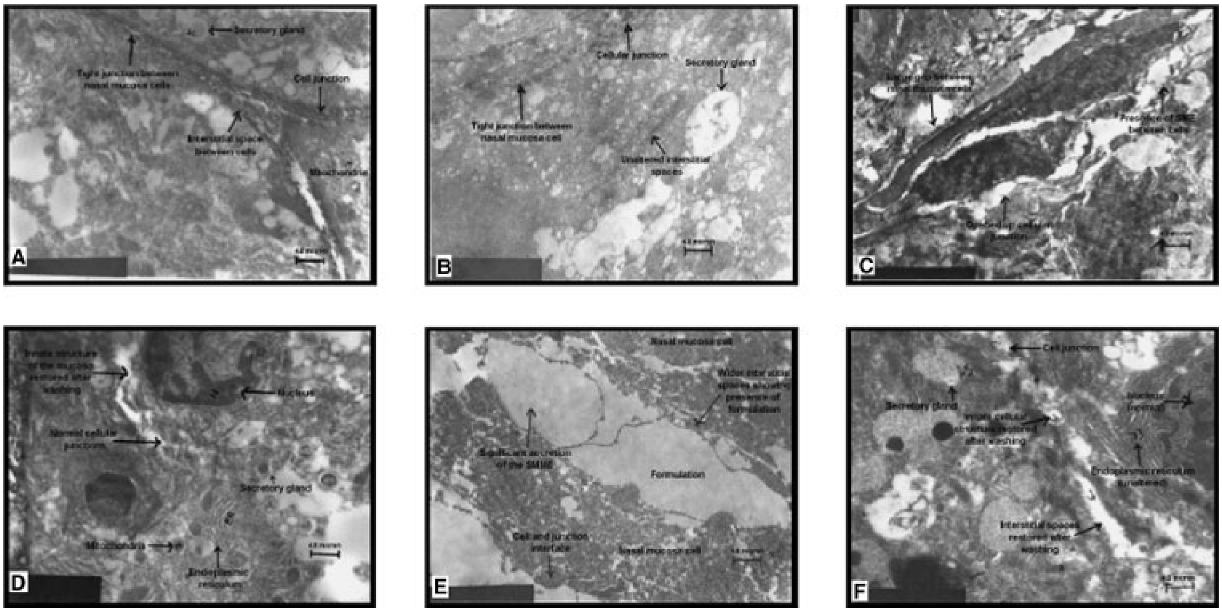

Figure 3. Electron micrographs of untreated and treated human nasal mucosa. (A) Nasal mucosa (untreated) showing presence of tight junctions between cells. (B) Nasal mucosa treated with CS indicating no change or uptake of CS. (C) Nasal mucosa treated with CME showing limited presence of formulation within interstitial space. (D) CME treated nasal mucosa washed with phosphate buffer retains innate cellular structure. (E) Nasal mucosa treated with CMME showing significant uptake within interstitial spaces between cells. (F) Nasal mucosa washed with phosphate buffer after CMME treatment. Innate cell structure is retained; mitochondria, endoplasmic reticulum, and normal interstitial spaces can be seen and no morphological changes in the normal cellular structures were observed.

CMME, respectively when evaluated for reduced/hydrolyzed (R/H) ^{99m}Tc and free ^{99m}Tc. The optimal $SnCl_2.2H_2O$ concentration was found to be 100 μg/mL at pH 7.0 with an incubation time of 30 min. ^{99m}Tc-CS/CME/CMME were found to be stable in normal saline solution and in rat serum up to 24 h (degradation $<5\%$ w/w). Bonding strength of ^{99m}Tc-CS/CME/CMME was also investigated by the DTPA challenging test, and the percent transchelation of the labeled complex was 1.82% w/w at 25 mM DTPA concentration, while at 100 mM, it increased to 4.03% w/w. The results suggested high bonding strength and stability of ^{99m}Tc-CS/CME/CMME. Thus these formulations were found suitable for biodistribution studies of the drug in rats.

Biodistribution studies[26] of ^{99m}Tc-CL formulations following i.v. administration (CME) and intranasal (CS, CME, and CMME) administration on Swiss albino rats were performed and the radioactivity was estimated at predetermined time intervals up to 8 h. The results obtained are recorded in Table 2. The brain/blood ratio of the drug at all time points for different formulations were also calculated and recorded in Table 2. The pharmacokinetic parameters were calculated from Figure 1 and recorded in Table 3.

After nasal administration of formulations, lower T_{max} values for brain (1–2 h) compared to blood (2–4 h) were observed. This may be attributed to preferential nose-to-brain transport following nasal administration. Moreover, following nasal administration of formulations, the drug concentrations in the brain were sustained for 2–3 h as evident from the plateaulike curve (Fig. 1B). The brain/blood ratios of the drug were found to be higher for formulations when administered intranasally (Tab. 2). This further confirms direct nose-to-brain transport.[34] The concentrations of the drug in brain following intranasal administration of CME and CMME were found to be significantly higher at all sampling time points compared to CME (intravenous) up to 8 h after nasal administration.

The substantially higher uptake in the brain with intranasal administration suggests a larger extent of selective transport of CL from nose-to-brain. Many researchers[35,36] have reported a unique connection between the nose and the brain and intranasal delivery of drugs to the brain bypassing the blood–brain barrier.[10,28] The $T_{1/2}$ of 5.0–5.6 h (blood), 3.2–3.6 h (brain), and K_{el} (blood) 0.11–0.14, 0.18–0.22 (brain) were observed irrespective of the routes of administration and the type of the formulations.

When CME i.v. was compared to CME nasal and CS nasal, significantly lower C_{max} and AUC were observed. The mucociliary clearance under normal circumstances rapidly clears the instilled formulation. However, when mucoadhesive agent was incorporated in the formulation (CMME), significant improvement in C_{max} and AUC was observed. Comparable AUC to CME i.v. was achieved with CMME nasal. This demonstrates the value of the mucoadhesive agent in prolonging the contact time of the formulation with the nasal mucosa.

Significantly higher AUC and C_{max} for CME nasal compared to CS nasal are attributed to microemulsion formulation.[27] The drug targeting efficiency (DTE (%)) and brain drug direct transport percentage (DTP (%)) were also calculated for nasally administered formulations and are shown in Table 4.

The CMME showed the highest DTE (%) and DTP (%) values among all the three formulations followed by CME and then CS. The twofold higher DTE (%) and twofold higher DTP (%) for CMME compared to CS show the benefit of the mucoadhesive microemulsion formulation. The higher DTE (%) and DTP (%) suggest that CMME has better brain targeting efficiency mainly because of substantial direct nose-to-brain transport. These findings are in congruence with the observations reported by Qizhi et al.[27] that microemulsion increases nose-to-brain uptake of the drugs.

In order to visualize brain uptake following intranasal and intravenous administrations of ^{99m}Tc-clonazepam microemulsion, we used a gamma scintigraphy camera to derive comprehensive biodistribution information. The gamma scintigraphy images in rabbits' 0.50 h post intravenous injection and intranasal administrations are shown in Figure 2. The presence of some radioactivity in the esophagus following i.n. administration could lead to absorption of a part of the formulation from gastrointestinal tract. The scintigraphy images were consistent with the results shown in Table 2 and high uptake of CMME into the brain was observed.

Electron micrographs of normal, formulation treated and washed human nasal mucosa are shown in Figure 3. The electron micrographs of nasal mucosa treated with various formulations revealed that CS treated nasal mucosa (Fig. 3B) showed presence of unaltered tight junctions which is similar to untreated nasal mucosa (Fig. 3A). However, higher uptake of CME (Fig. 3C) was found compared to CS. Significant accretion of CMME (Fig. 3E) compared to CME and presence of formulation was noticed within the junctions of nasal mucosae cells. The nasal mucosa washed (Fig. 3D and F) after formulation treatment was found to restore the innate cellular structure, and normal endoplasmic reticulum, mitochondria, and nuclei were observed. The electron micrographs revealed that the innate structure of mucosa is restored after formulation treatment and washing suggesting reversal of dilation of tight junctions. Moreover, the results also demonstrated the presence of a high quantity of CMME within the interstitial spaces of tight junctions of nasal mucosae cells indicating paracellular mode of transport of CMME. These findings corroborate observations reported by Gavini E and coworkers that on exposure of nasal mucosa to formulation containing mucoadhesive agent showed opened tight junctions.[17]

CONCLUSIONS

In this investigation, mucoadhesive microemulsion of clonazepam was successfully prepared and demonstrated in rats to deliver clonazepam to the brain rapidly and more effectively with intranasal administration. Accumulation of formulation within interstitial spaces and transport of drug to the brain may be due to stretching of tight junctions within the nasal mucosa. The studies suggest intranasal delivery of clonazepam to be promising. However, benefits to risk ratio and clinical intricacies need to be scientifically established for its suitability in clinical practice in the management of emergencies of status epileptics.

List of Abbreviations

Abbreviations	Description
CL	clonazepam
CS	clonazepam solution
CME	clonazepam microemulsion
CMME	clonazepam mucoadhesive microemulsion
^{99m}Tc	technetium

^{99m}Tc-CS	radiolabeled (99m-technetium) clonazepam solution
^{99m}Tc-CME	radiolabeled (99m-technetium) clonazepam microemulsion
^{99m}Tc-CMME	radiolabeled (99m-technetium) clonazepam mucoadhesive microemulsion

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