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REVIEW ARTICLE

Evaluation of Brain Targeting of Drugs after Administered Intranasally

Meghana S. Kamble[*1], Sandeep M. Dange[1], Kishor K. Bhalerao[1], Pravin D. Chaudhari[1], Ashok V. Bhosale[2].

[1]P. E. Society's Modern College of Pharmacy, Nigdi, Pune-411 044, Maharashtra, India

[2]P. D. E. A.'s S. G. R. S. College of Pharmacy, Saswad, Pune, Maharashtra, India

ABSTRACT

Intranasal route has potential to target brain and possesses several advantages over parenteral and oral routes in treating CNS disorders such as stroke, Parkinson's disease, multiple sclerosis, Alzheimer's disease, epilepsy and psychiatric disorders. Nasal drug delivery is attracting attention of many researchers to develop efficient drug delivery systems to target brain using novel drug delivery systems. A number of in-vivo, ex-vivo and in-vitro methods have been used to study and evaluate nose-to-brain drug delivery system. The present review discusses the currently available methods to evaluate brain uptake of drug after intranasal administration.

KEY WORDS: Intranasal, Olfactory, in vivo-ex vivo evaluation.

INTRODUCTION:

Various drugs have been tried by researchers to target CNS through intranasal route. Various in vivo models have been used to evaluate the drugs targeting CNS by administering via intranasal route. This review covers currently used methods of nose to brain drug delivery. Various bioanalytical methods used for determination of drugs in biological fluids are Ultra performance liquid chromatography-Mass spectrometry (UPLC-MS), High performance liquid chromatography-Mass spectrometry (HPLC-MS), Gas chromatography-Mass spectrometry (GC-MS), Gas chromatography-Nitrogen phosphorus detector (GC-NPD), High performance liquid chromatography-Ultraviolet detection (HPLC-UV), High performance liquid chromatography-Fluorescence (HPLC-FL), Reverse phase-High performance liquid chromatography (RP-HPLC). Various in vivo & ex vivo methods used for determinations of brain targeting by intranasally administered drugs are discussed in this article.

1. BRAIN UPTAKE STUDY[1, 2]:

A. Isolation of brain & determination of drug in brain:

Three rats for each formulation per time point are used in the studies.

Group 1 receives drug solution in a specific dose dissolved in 1 ml of sterile isotonic solution which is administered intravenously (IV).

Group 2 receives drug solution in a specific dose dissolved in 100µl. of normal saline solution which is administered intranasally (IN).

Group 3 receives drug delivery system dissolved in 100 µl normal saline solution which is administered (IN).

The rats are euthanized at scheduled times by cervical dislocation after sedating them with chloroform to collect brain tissue. The skull is cut open and brain is carefully excised. It is quickly rinsed with saline and blotted with filter paper to get rid of blood-taint and macroscopic blood vessels as much as possible. Brain samples are obtained after 15, 30, 60, 120, and 480 min respectively, after administration of dose by different routes. The concentrations of drug in brain tissues are analyzed by suitable validated bioanalytical method.

B. Microdialysis Method by probe implantation into brain

Probe implantation into brain and microdialysis procedure[3,4]:

The study is performed on male Sprague – Dawley rats, weighing approximately 350 g, with free access to food and water. The animals are anaesthetized by a subcutaneous injection of a hypnorm / dormcium / water mixture (1:1:2) and placed on a heating plate to maintain a normal body temperature. The arteria carotis is cannulated by a heparin-filled polyethylene catheter for blood sampling. A microdialysis probe is inserted into the left vena jugularis by use of a guide cannula and the head of the probe is fixed to the breast musculature. The animal is then placed in a stereotaxic frame and the skull is exposed by an incision in the scalp. A stainless steel screw is placed in a 0.5 mm hole drilled in the skull. Two 1mm holes are carefully drilled through the skull by a trephine drill at 0.8 mm anterior and ± 2.7 mm laterally relative to the Bregma. The Dura is carefully perforated by a needle and microdialysis guides fit with dummies are implant through the holes and fixed to the skull with dental cement, secured by the stainless steel screw. After 30 min the

[*]***Corresponding author:*** Meghana S. Kamble ***/ Email:*** formeghana@yahoo.com

cement is hardened, the dummies are removed and the probe is inserted through the guides. The ventral position of the tip of the probe is 4.7mm relative to Bregma, leaving the entire membrane surface of the brain probes in left and right striatum, respectively. The integrity of the blood–brain barrier following the implantation procedure is previously found to be sufficient. After implantation, the microdialysis probes are perfused with blank Ringer's Solution for 60 min before either intravenous or unilateral nasal administration is given. The intravenous formulation is injected in the tail vein. Nasal administration is given 20mm into the cavity as a droplet on the mucosa in the right nostril by inserting a soft catheter fitted to a 25 µl Hamilton microsyringe. By using this technique it is possible to deposit the formulation at or close to the olfactory epithelium. The rats are kept on the abdominal side throughout the study and no modifications are made to the naso-palantine duct in order promote normal function of the nasal cavity, i.e. ciliary movement and drainage of the formulation. Fractions of dialysate from the microdialysis probes are sampled with 10 min intervals for the first 60 min and with 15 min intervals from 60 to 180 min after administration. Blood samples are taken from the arterial catheter at 0, 30, 60, 90, 120 and 180 min. The blood is sampled in heparin-coated vials and centrifuged at 1700 × *g* for 10 min to isolate the plasma, which is stored at −20^{0}C until analysis**.**

2. PHARMACOKINETIC STUDY:

A. In vivo study:

Drug absorption study by nasal cavity isolation and jugular vein cannulation[3]:

The rats are anesthetized with an intra-peritoneal injection of urethane. During the experiment, body temperature is maintained at 37^{0}C under an infrared lamp. After an incision is made in the neck, the trachea is cannulated with a polyethylene tube to maintain respiration. Another PE-200 tube is inserted through the esophagus towards the posterior part of the nasal cavity and ligated. The passage of the nasopalatine tract is sealed with an adhesive agent to prevent the drainage of the solution from the nasal cavity to the mouth. A polyethylene tube is inserted into the jugular vein for blood sampling. After administration of drug to nasal mucosa, blood samples are collected at suitable time intervals and analyzed by suitable validated bioanalytical method.

B. Nasal absorption studies of administered by spraying onto nasal mucosa[5]:

White rabbits (3.0–4.0 kg) are used for nasal and intravenous administration with a wash out period of 2 weeks. Rabbits are weighed and restrained in rabbit restrainers before the experiment. For intravenous administration, an intravenous infusion of drug injection is delivered through the marginal ear vein of rabbit at specific dose for over 20 sec. For intranasal administration, 100µl of the nasal formulation is given in each nostril of rabbit using a metered-dose pump spray device for within 5 sec. Blood sampling (0.5 ml) is done before dosing and at 2, 5, 10, 20, 30, 45, 60, and 120 min after dosing via an artery catheter set up at the rabbit ear. Blood samples are anti coagulated with EDTA and centrifuged at 3000 rpm for 15 min. The plasma is separated and stored at −20 °C until analysis.

C. Nasal absorption of drug administered by applying to instillation of drops on to nasal mucosa[1, 2]:

Three rats for each formulation per time point are used in the studies.

Group 1 receives drug solution in a specific dose dissolved in 1 ml of sterile isotonic solution which is administered IV.

Group 2 receives drug solution in a specific dose dissolved in 100µl. of normal saline solution which is administered IN.

Group 3 receives drug delivery system dissolved in 100 µl normal saline solution which is administered IN.

The rats are euthanized at scheduled times by cervical dislocation after sedating them with chloroform to collect blood. The blood samples are collected from retino orbital vein present in the eye in precoated EDTA tubes. The blood samples are obtained after 15, 30, 60, 120, and 480 min, respectively, after administration of dose by different routes. The concentrations of drug in blood are analyzed by suitable validated bioanalytical method.

PHARMACOKINETIC ANALYSIS[1, 2]:

Plasma concentration–time profiles of drug after intranasal and intravenous delivery are evaluated by pharmacokinetic software (PK Functions for Microsoft Excel, Pharsight Corporation, Mountain View, CA). The maximum plasma concentration of drug (C max) and the time required to reach the maximum concentration (T max) are obtained directly from the actual plasma profiles. The area under the curve between 0 and 480 min is calculated by the linear trapezoidal method. Brain targeting efficiency is calculated using two equations mentioned below.

Drug targeting efficiency (DTE) represents a time-average partitioning ratio.

$DTE = AUC_{brain} / AUC_{plasma}$

Drug targeting efficiency (DTE %) that represents time average partitioning ratio is calculated as follows:

Drug targeting efficiency (DTE %) =

$(AUC_{brain} / AUC_{blood})$ IN / $(AUC_{brain} / AUC_{blood})$ IV ×100.

Where, IN.- intranasal, IV.- intravenous.

Nose to brain direct transport percentage (DTP %) is calculated as follows:

Direct transport percentage (DTP %) =

$(B_{i.n.} – B_x) / B_{i.n.} \times 100$

Where $B_x = (B_{i.v.} / P_{i.v.}) \times P_{i.n.}$,

B_x is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration;

$B_{i.v.}$ is the $AUC_{0–480}$ (brain) following intravenous administration;

$P_{i.v.}$ is the $AUC_{0–480}$ (blood) following intravenous administration;

$B_{i.n.}$ is the $AUC_{0–480}$ (brain) following intranasal administration;

$P_{i.n.}$ is the $AUC_{0–480}$ (blood) following intranasal administration.

DATA ANALYSIS:

Absolute concentrations in CSF are calculated from the concentrations in the dialysates using the following equation:

C = Cd/ R

Where R is the in vivo relative recovery.

The area under the plasma concentration–time curve AUC_{plasma} value and CSF concentration– time curve AUC_{CSF} value are calculated using the trapezoidal rule. The degree of drug targeting to CSF after intranasal administration can be evaluated by the drug targeting index (DTI), which can be described as the ratio of the value of AUC_{CSF} / AUC_{plasma} following intranasal administration to that following intravenous injection.

The higher the DTI is, the further degree of drug targeting to CSF can be expected after intranasal administration.

CALCULATION AND STATISTICS:

All plasma concentration data are dose- and weight-normalized, and then analyzed using Win-N online noncompartmental model (Scientific Consulting, Apex, NC). The area-under-the curve ($AUC_{0–t}$) is determined by the linear trapezoidal method. The C_{max} following IV injection is estimated by extrapolating the initial plasma drug concentration–time curve to the Y-axis at time zero (C_0), and the C_{max}, C_{2min}, and t_{max} values of the nasal administration are read directly from the concentration-time profile. The $t_{1/2}$ is calculated by fitting the data of the terminal portion of the pharmacokinetic profile by a log linear regression equation. The absolute bioavailability (F %) of nasal administration from microemulsions is calculated using the following Eq.

$F (\%) = (AUC_{0–t, nasal} \times Dose_{IV}) / (AUC_{0–t, IV} \times Dose_{nasal}) \times 100$

Statistical analysis is performed utilizing standard method; Student's t-test is employed for calculating the significance (P = 0.05). Qualitative localization and biodistribution studies by confocal laser scanning microscopy (CLSM) Rhodamine-123 (ROD-123) fluorescent dye is used to determine the localization of drug Nanoparticles in brain and other organs qualitatively. ROD-123 is widely used in cerebral studies and it is unable to cross the BBB. For the preparation of ROD-123 loaded chitosan Nanoparticles, 0.9 ml of Rhodamine 123 (20 mg/ml in ethanol) is dissolved in 10 ml of chitosan solution (0.175%, w/v) followed by its cross linking with 4 ml of 0.2% (w/v) TPP solution at room temperature with constant stirring at 800–900 rpm for 30 min. The resultant NPs are concentrated by centrifugation at 15000 rpm at 10^0C for 40 min.

For bio-distribution studies animals are divided into two groups, each composed of 3 animals.

Group 1: receives ROD-123 loaded Nanoparticles intravenous in the dose of 0.836 mg/day dissolved in 1 ml of sterile isotonic solution by tail vein.

Group 2: receives ROD-123 loaded chitosan Nanoparticles intranasal at the dose of 0.836 mg/day dissolved in 100µl of normal saline solution.

Then the animals are sacrificed at fixed time point of 20 min, 60 min and 120 min to localize the Nanoparticles in different organs (i.e. brain, liver, lung and spleen) and the isolated organs are washed with Ringer's solution. Cerebellum is separated from brain after dissection of right, left and frontal encephalon. Each tissue is cut in its thickness by a microtome into 5µm slides. The slides are then fixed in 4% (w/v) formaldehyde solution and stored at 4^0C before confocal laser scanning microscopy (CLSM) studies. In order to demonstrate the location of Nanoparticles, tissue slides are stained for 10 min with 50µl (250 ng/ml) of DAPI (4-6-diamidino-2-phenylindole; Sigma Aldrich, Mumbai, India) solution, which is known to form fluorescent complexes with natural double-stranded DNA, and observed using a fluorescence microscope (Olympus FluoViewTM FV 1000, CA, USA) with double band, for DAPI and ROD-123. The red fluorescent spots due to the fluorescent dye ROD-123 are considered as the visible markers of the drug embedded into Nanoparticles.

3. PHARMACODYNAMIC STUDIES:

A. Modified forced swim test[2]:

Antidepressant activity is determined by forced swim test on chronic depression induced rats.

Animals are divided into five groups with three animals in each group.

Group 1 is considered as naive group which neither received any drug nor depression is induced in it.

Group 2 rats are given marketed tablet drug solution at specific dose.

Group 3, 4 and 5 rats are given nasal formulation in each nostril with the help of a microlitre syringe attached to polyethylene tubing having 0.1 mm internal diameter. The polyethylene tubing is inserted 7 mm inside the nostril.

Group 3 rats are kept as control and given saline solution in each nostril for 15 days.

Group 4 rats have been given pure drug solution at the specific dose dissolve in 100 µl of normal saline solution for 15 days.

Group 5 rats have given drug formulation dissolved in 100 µl normal saline solution 1 h prior to intranasal administration for 15 days. Rats are placed in a cylindrical glass tank (46 cm tall and 20 cm in diameter) filled to a depth of 30 cm water (25 ± 2 ^{0}C) individually for 6 min training and are then removed and dried. The water depth of 30 cm allowed the rats to swim or float without their hind limbs or tails touching the bottom of the tank. The 6 min training is also given 30 min after administration of dose in each group for 15 days. After 5 h (on 15th day) animals again are re-exposed to the swimming in a similar environment for 6 min and behavioral climbing time, swimming time and immobility time has been record for 300 s and compare with group 3 control (untreated but trained, i.e. depressed) and group 1 naive (neither untreated nor trained, i.e. non depressed animals).

B. Measurement of locomotor activity[2]:

The locomotor activity is recorded for 5 min using photoactometer on 15th day for all the five groups as mentioned above. Each animal is observed in a closed square (30 cm × 30 cm) area equipped with infrared light sensitive photocells using digital photoactometer. Locomotor activity is expressed in terms of total photo beam counts for 5 min per animal. The apparatus is place in a darkened, light; sound attenuated, and ventilated testing room.

C. Eddys Hot plate method in mice for analgesic effect[6]:

This method is used to assess centrally mediated analgesic effect of drug. The centrally acting analgesic drug is used as positive control group. In this experiment two groups (n=6) of Swiss albino mice (20-25g) are place on hot plate maintained at room temperature for 15 min. Food is withdrawn on the preceding night of experiment.

Group 1: Normal control

Group 2: Given drug

Each animal is then individually place on Eddys hot plate at 55^{0}C. The nociceptive response such as licking paws or jumping off is determined at 15, 30, 45, 60 and 90 min after administration of test drug.

D. Rota-rod treadmill[7]:

Rota-rod treadmill (Yuanhua, Chengdu, Sichuan, China) consisting of a roller with 8 cm diameter suitably machined to provide grip and a power source to turn the roller is used. Five circular separators divided the rod into equalsized compartments (8 cm length each), enabling four rats to be on the treadmill simultaneously. The central drum surface is of engineering plastics with fine grids, which provided a non-slip surface for animals. The rotation drum is driven by a heavy duty D.C. motor. Accelerating Rota-rod might be set at any speed from 0 to 50 r.p.m. Drive speed is practically unaffected by voltage variations, friction or wear, which ensures that screening runs could be repeated in constant operating conditions. The time spent by each rat on the Rota-rod is measured by timers, which is detected when the rat falls off the treadmill onto the plate below. The balance beam rod of 160 cm in length and a diameter of 2.5 cm. A plastic platform (7 cm × 4 cm) is set at one end of the rod as the start, and a black plastic box (15 cm × 15 cm × 8 cm) is at the other end of the rod as a nest for motivating the animal crossing from the beam. The apparatus is suspending 90 cm above a cushion, which protected the fallen animals from injury, and 50 cm from a wall. The time taken to traverse the beam is recorded.

Open field tests are first conducted following microinjections for assessment of effects of histamine and GABA on motor and general behavior. On the other hand, the behavioral tests for motor balance and coordination are divided into five stages, which are named stages 0, 1, 2, 3, and 4, respectively. Each of them included five trials, which consists of a task on the accelerating Rota-rod followed by the balance beam test 30 sec later. To reduce stress and fatigue, the animals are allowed to take a rest for at least 90 sec between trials. Before microinjections and behavioral tests, each animal is trained daily in at least 10 trails for three consecutive days in order to achieve a stable performance on the Rota-rod and beam. The stage 0 tests are conducted just before the microinjections and all these tests are started at approximately same time. Then, the stage 1 tests are performed once the injections finished. The stage 2 tests are carried out 4 h after the

injections, and the stage 3 tests are executed 24 h after the injections. Only some of group intravenous rats go through stage 4. Each stage test lasts not more than 1 h.

4. EX VIVO STUDY:

Fresh nasal tissue is carefully removed from the nasal cavity of sheep obtained from the local slaughter house. Tissue sample is inserted in Franz diffusion cells displaying a permeation area of about 0.785 cm^2. 20 ml of phosphate buffer saline (PBS) pH 6.4 at 34^0C is added to the acceptor chamber. To ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 is bubbled through the system. The temperature within the chambers is maintained at 34 ^0C. After a pre-incubation time of 20 minutes, pure drug solution and formulation equivalent to drug is placed in the donor chamber. At predetermined time points, 1ml samples are withdrawn from the acceptor compartment, replacing the sampled volume with PBS pH 6.4 after each sampling, for a period of 4 hours[8]. The samples withdrawn are filtered and used for analysis. Blank samples (without drug) are run simultaneously throughout the experiment to check for any interference. The amount of permeated drug is determined using a suitable validated analytical method. This ex vivo study gives an idea about permeation of drug from a particular drug delivery system through nasal mucosa. Ex vivo study also is carried out using rabbits, bovine, porcine tissues.

5. NASAL TOXICITY:

Histopathological evaluation of tissue incubated in PBS (pH 6.4) after collection is compared with tissue incubated in the diffusion chamber with formulation. Tissue is fixed in 10% buffered formalin (pH 7.2), routinely processed and embedded in paraffin. Paraffin sections (7 µm) are cut on glass slides and stained with hematoxylin and eosin (HE). Sections are examined under a light microscope, to detect any damage to the tissue during in vitro permeation by a pathologist blinded to the study.

NASAL MUCOTOXICITY[9, 10]:

The mucosa toxicity evaluation is conducted using toad palate and rat nasal mucosa. In the experiment on toad palate, the upper palate mucosa of toads in each group is respectively exposed to 0.5 ml of saline, drug, drug formulation and positive control for 1 hour, rinsed with saline, and dissected and examined under an optical microscope. The duration time of the ciliary movement is recorded and pictures are taken by a camera. In the experiment on rat nasal mucosa, formulation, drug-formulation or saline is administrated successively for one week to the nostrils of each group (n = 3) using a polyethylene 10 (PE 10) tube attached to a microliter syringe. The rats are sacrificed and the nasal mucosa is harvested at 24 hours after the last administration. Immunohistochemical staining is performed on paraffin sections (5-µm thick) of nasal mucosa for neuron specific enolase (NSE) examination. In that sections are incubated with rabbit anti-nerve-specific enolase polyclonal antibody, performed with SABC kit, and finally stained with DAB. Images of sections are obtained using a microscope.

NASAL CILIOTOXICITY[10, 11]:

Nasal ciliotoxicity studies are carried out using in situ toad palate model. Upper palate of toad exposed and treated with 0.5 ml test formulations for 30 min, is then rinsed with saline. The palate is isolated and the mucocilia are examined with a 400-fold Motic DMBA 450 microscope. The beat of cilia is observed and the lasting time of the ciliary movement is recorded. The relative percentages of lasting time of the ciliary movement of the test formulations are calculated as the ratios of the lasting time of ciliary movement after being treated with test formulations to that being treated with physiological saline. Negative and positive controls are also used.

CONCLUSION:

This article attempts to review currently available in-vivo & ex-vivo animal model for intranasal drug delivery system. As the scope of novel drug delivery system is widening, the use of animal model in early stages of drug development is increasing. A number of in vivo-ex vivo models have been used currently for determining the drug targeting CNS via nasal route. Every method has its own pros & cons. It is important to know suitability of method for particular study, the selection dependent on validation & relevance of the method. Though in certain cases ex vivo studies replacing in vivo studies but it can't replace in vivo studies completely. Ex vivo studies give an idea about drug permeation & toxicity of formulation under study. Further efforts are required to study in vivo- ex vivo correlation of data.

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