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Nose-to-brain transport of melatonin from polymer gel suspensions: a microdialysis study in rats

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Abstract

Purpose—Exogenous melatonin (MT) has significant neuroprotective roles in Alzheimer's and Parkinson's diseases. This study investigates the delivery MT to brain via nasal route as a polymeric gel suspension using central brain microdialysis in anesthetized rats.

Methods—Micronized MT suspensions using polymers [carbopol, carboxymethyl cellulose (CMC)] and polyethylene glycol 400 (PEG400) were prepared and characterized for nasal administration. *In vitro* permeation of the formulations was measured across a three-dimensional tissue culture model EpiAirway™. The central brain delivery into olfactory bulb of nasally administered MT gel suspensions was studied using brain microdialysis in male Wistar rats. The MT content of microdialysis samples was analyzed by high performance liquid chromatography (HPLC) using electrochemical detection. The nose-to-brain delivery of MT formulations was compared with intravenously administered MT solution.

Results—MT suspensions in carbopol and CMC vehicles have shown significantly higher permeability across EpiAirway™ as compared to control, PEG400 ($P < 0.05$). The brain (olfactory bulb) levels of MT after intranasal administration were 9.22, 6.77 and 4.04-fold higher for carbopol, CMC and PEG400, respectively, than that of intravenous MT in rats. In conclusion, microdialysis studies demonstrated increased brain levels of MT via nasal administration in rats.

Keywords

Melatonin; bioadhesive polymers; brain microdialysis; cutaneous microdialysis; nasal delivery

Introduction

Melatonin (MT) is an indole amide neurohormone secreted by the pineal gland. It plays a critical role in the synchronization of body rhythms with night and day cycles. MT has been in use in the management of sleep disorders such as delayed sleep syndrome, jet lag and shift work syndrome and migraine headaches (Sánchez-Barceló et al., 2010). MT has

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demonstrated neuroprotective and antioxidant properties against amyloid β -protein mediated oxidative injury *in vitro* (Pappolla et al., 1997) as well as in *in vivo* in transgenic mice (Matsubara et al., 2003). In addition, it has been shown that MT inhibits the formation of beta-sheets and amyloid fibrils (Pappolla et al., 1998). Exogenous MT has been investigated for its neural protective roles in the treatment of Alzheimer's and Parkinson's diseases (Pappolla et al., 2000; Hayter et al., 2004; Kaur et al., 2008; Wang, 2009). However, MT has very low oral bioavailability (below 20%) due to an extensive first-pass hepatic and gut wall metabolism, an erratic pharmacokinetic profile and a short biological half-life of 45 min (Lane and Moss, 1985; Yeleswaram et al., 1997; DeMuro et al., 2000). Transdermal patches (Kanikkannan et al., 2004; Priano et al., 2007), topical creams (Fischer et al., 2001), nanoparticles (Hafner et al., 2009) and liposomes (Dubey et al., 2007) have been investigated to improve systemic bioavailability of MT. However, the systemically delivered MT cannot cross blood-brain barrier to provide adequate brain levels. The nasal delivery has direct access to brain and therefore can be considered as a promising route for improving brain levels of MT. However, due to the low aqueous solubility of MT and short residence time in the nasal cavity, its delivery presents several problems. Various delivery methods can be used for the enhancement of drug concentrations in the brain such as local intracerebral implants, or disruption of the blood-brain barrier by infusion of hyperosmotic solutions or vasoactive agents prior to systemic administration of the drugs. Being highly invasive, these methods are most appropriate for short-term treatments, where a single or infrequent exposure to a drug is required, but not an ideal method for long-term treatments (Illum, 2003). One approach for improving drug absorption has been the use of bioadhesive polymers to increase the residence time and to sustain the drug absorption in the nasal cavity (Harris et al., 1988a; Harris et al., 1988b; Morimoto et al., 1991; Charlton et al., 2007).

Although intranasal administration is used primarily for systemic delivery of drugs, the nasal cavity houses the olfactory nerve cells that are in direct contact with the central nervous system (CNS). This unique connection of nose and the CNS has been investigated to deliver therapeutic agents via nose to the brain thus the blood-brain barrier can be circumvented (Graff and Pollack, 2005). The nose-to-brain delivery is a novel noninvasive method to rapidly deliver drugs directly from the nasal mucosa to the brain and spinal cord with the aim of treating CNS disorders while minimizing systemic exposure (Dhuria et al., 2010). Small molecular weight compounds, cocaine, benzoylecgonine (Chow et al., 2001), dihydroergotamine (Wang et al., 1998), prostaglandin analogs (Yamada et al., 2007), Zolmitriptan (Jain et al., 2010) and renin inhibitor (Kararli et al., 1992) have been reported to enter the CNS via nasal delivery in animals. In addition to these, several recent reports have shown that therapeutics given by the intranasal route are delivered to the CNS and have the potential to treat neurological diseases and disorders (Frey, 2002; Costantino et al., 2007; Hanson and Frey, 2008; Vaka and Murthy, 2010). Intranasal delivery can reduce systemic exposure for large molecular weight drugs such as insulin, which can directly reach the brain from the nasal cavity without altering systemic blood levels of insulin or glucose in humans (Born et al., 2002). Intranasal insulin has been shown to improve memory in normal adults and patients with Alzheimer's disease (Reger et al., 2008a; Reger et al., 2008b).

Several methods have been used to study the uptake of substances into the brain after intranasal administration, including cerebrospinal fluid (CSF) sampling by cisternal puncture, biopsies of brain tissues and autoradiography (Merkus et al., 2003; van den Berg et al., 2004). We have utilized microdialysis sampling of CSF from the olfactory bulb for determining MT concentration. MT was delivered intranasally as a polymer gel suspension and MT levels in both brain and subcutaneous tissue was measured by microdialysis sampling in Wistar rats.

Materials and methods

Materials

Carbopol 934P NF was a gift from BF Goodrich (Cleveland, OH). MT and carboxymethyl cellulose (CMC, medium viscosity grade) were procured from Sigma-Aldrich (St Louis, MO). EpiAirway™ 3-D culture tissues were procured from MatTek Corporation (Ashland, MA). Microdialysis probes (CMA12/4 for brain and CMA20/10 for blood), pumps (CMA/100), tubing (i.d. 0.4 mm) and other accessories were obtained from CMA/Microdialysis AB (Solna, Sweden). The stereotaxic frame was obtained from Harvard Instruments (Holliston, MA).

MT formulations

MT was subjected to micronization by ball-milling process. Briefly, 2 g of MT was placed in the grinding jar with 12 mm stainless steel balls of the ball mill (Type MM 200, Glen Mills Inc. Clifton, NJ). Milling was carried out for 8 hours with a 30 min cooling time interval at each hour during milling. The milled drug was passed through # 325 mesh sieve (U.S. standard sieve, Newark, NJ) which provided 100% particles below 45 µm. To confirm the physical/chemical stability of MT due to milling, the product was subjected to differential scanning calorimetry (DSC) and high performance liquid chromatography (HPLC) analyses. Nasal formulations of micronized MT (1% w/v) was prepared with 1% CMC or 0.125% carbopol (adjusted to pH-6.4) and 0.1% w/v Tween-80 (as a wetting agent) in de-ionized water.

Rheological and droplet size characterization of MT formulations

The rheological behavior of the formulations was investigated using a small sample adapter attached to the LV-DV-III Brookfield viscosity meter. Measurements were performed at 25°C and the temperature was maintained using water bath that circulated fluid in the sample jacket. Rheological behaviors of the formulations were determined by plotting the viscosity as a function of shear rates. Rheological constants were obtained from the regression line, using Rheocal software (Middleboro, MA).

The experimental methods for droplet size characterization were described in more detail in a previous publication (Dayal et al., 2004). In brief, droplet size analysis of nasal aerosols was conducted by laser diffraction using a Malvern Spraytec® with RT Sizer software. InnovaSystems eNSP station (Moorestown, NJ) was used to actuate nasal pumps. The first five actuations from spray container enclosing the test formulation were fired into waste to prime the device, followed by one test actuation. Drop size distribution (DSD) was measured after one actuation, and each formulation was tested six times. DSD measurements were conducted at 3 cm from the laser beam. All measurements were made at room temperature (~23°C). Data was reported as volume diameter defined by 10%, 50% (volume median), and 90% of the cumulative volume undersize (Dv10, Dv50, and Dv90).

Solubility studies of MT in the formulations

A saturated solution of MT was made with each vehicle by agitating on an environmental shaker at $23 \pm 0.1^\circ\text{C}$ for 48 h. After equilibration, the suspension was filtered through a 0.2-µm nylon syringe filter, diluted suitably and the MT content was assayed by HPLC.

In vitro drug release studies

In vitro drug release studies of MT (molecular weight, 232.2 Da.) were carried out using Franz diffusion cells (Hanson Research, NJ). Dialysis membranes (6000–8000 Da. molecular weight cut off) were mounted and clamped between the receiver and donor

compartments of the diffusion cells. 100 μ l of the test formulation was placed in the donor cap that had a 0.634 cm² diffusion area. The receptor compartment was filled with de-ionized water (5 ml) and the temperature maintained at 37°C by circulating thermostatically controlled water through a jacket surrounding each cell body. The contents were stirred continuously with a magnetic stirrer at 600 rpm. At intervals (5, 15, 30, 45, 60, 90, 120, 240, 360 and 480 min), the receptor compartment was sampled, emptied and replaced with fresh medium. All *in vitro* drug release studies were performed in triplicate and samples were assayed for MT using a HPLC.

In vitro permeability studies

Permeability studies were performed using three-dimension human-derived tracheal/bronchial epithelial cells cultures (EpiAirway™) with 1 cm² diffusion area (MatTek Corporation, Ashland, MA). These tissues were well utilized as permeation barriers of nasal membranes as reported in earlier investigations (Agu et al., 2004, Agu et al., 2006, Babu et al., 2008). The tissues grown on the deep snap wells along with the modified 6-well plates provided a secure donor and receiver compartments. The receiver chamber was filled with 6 ml of Dulbecco's phosphate buffered saline. The diffusion plate setup was placed in a rotator water bath that was maintained at 37°C as illustrated in Figure 1. The rotator water bath was set at 75 rpm, which provided sufficient agitation of the buffer solutions and provided sink conditions. Before each experiment, the tissue was equilibrated in Dulbecco's phosphate buffered saline for 30 min. The entire volume (6 ml) was collected from the receiver compartment at intervals (0, 15, 30, 45, 60, 90, 120, 180 and 240 min) and replaced by fresh pre-warmed buffer solution. The amount of MT in the samples was analyzed by HPLC with electrochemical (EC) detector. The integrity of the monolayer of nasal cells was determined by the transepithelial electrical resistance (TEER) measurement using a volt ohm meter (Millipore, Millicell®-ERS, Burlington, MA). Cell viability of tracheal/bronchial epithelial cells cultures following diffusional studies were determined by methylthiazoletetrazolium (MTT) assay according to the manufacturer's protocol.

Animals

Adult Male Wistar rats, 230–270 g, 6–8 weeks of age were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained in the College of Pharmacy, Florida A&M University animal housing facilities. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Florida A&M University.

Nasal and intravenous delivery of MT

The rats were anesthetized by intraperitoneal injection of Urethane (1.5 g/kg). The animals were then placed in a stereotaxic frame and the skull was exposed by an incision on the scalp. Two holes (1 mm diameter) were carefully drilled through the skull by a trephine drill at AP: + 8.2, LR: +/-1, DV: -1 relative to the Bregma. The two holes represent the position of the right olfactory (LR: +1) or the left olfactory bulb (LR: -1). The Dura was carefully perforated with a needle and the microdialysis guides fitted with dummies were implanted through the holes and fixed to the skull with dental cement. After the cement was hardened in 20 min, the dummies were removed and the probes inserted through the guides. The ventral position of the tip of the CMA12/4 probe was 1 mm relative to Bregma, leaving the entire membrane surface of the probes in left and right olfactory bulb, respectively. The integrity of the blood-brain barrier following the implantation procedure was previously found to be sufficient (de Lange et al., 1999). After implantation, the microdialysis probes were perfused with blank artificial CSF for 40 min before either intravenous or unilateral nasal administration to achieve equilibrium between perfusate and physiological environment. The intravenous MT formulation (0.5 mg/kg) was injected in the tail vein. Nasal administration of test MT formulations (0.5 mg/kg in 13–17 μ l of 1% w/w MT

suspension) into the cavity was achieved as droplets by inserting a soft catheter fitted to a 0.5 ml syringe (30 gauge). The formulations were dispensed over a period of 60 s from a catheter that produced very tiny droplets, based on the diameter of the needle. The catheter was marked with a length of 5 mm inserted into the nostril until the mark was reached in order for consistent dosing in the rats. Although we would like to target the olfactory epithelium, the rat's nasal cavity is approximately 50% lined by olfactory epithelium with no anatomical transitioning from olfactory to respiratory (Gross et al., 1982). We conducted preliminary work using eosin Y stain for optimal dosing into rat's nasal cavity; however, no studies were conducted to distinguish the respiratory or olfactory epithelium in rats. The rats were kept on their supine position throughout the study and no modifications were made to the naso-palantine duct in order to promote normal function of the nasal cavity, i.e., ciliary movement and drainage of the formulation. The dose was administered ipsilateral to the placement of the probe. For the probe located in the left olfactory bulb, the dose was administered in the left nostril. Fractions of dialysate from the microdialysis probes were sampled with 10-min intervals for 180 min and collected in borosilicate vials containing 5 μ l of 0.01 M perchloric acid/100 μ M Ethylenediamine tetraacetic acid (EDTA).

Systemic levels of MT were determined by subcutaneous microdialysis. Implantation of CMA20/10 probes in the abdominal skin area was performed as described in earlier reports (Lindberger et al., 1998; Graumlich et al., 2000; Mathy et al., 2001) and samples times were synchronized along with brain samples times. After implantation, the probes were perfused with Ringer's solution for 40 min before either intravenous or unilateral nasal administration.

Microdialysis recovery determination

The *in vitro* recovery by gain was tested by placing brain microdialysis probes (CMA12/4) in 50 μ g/ml of MT in Ringers solution and perfusing the fluid at 2 μ l/min. Six dialysate samples were collected from each probe at 10-min intervals after initial equilibration for 20 min. Similarly, *in vitro* recovery of vascular (subcutaneous) microdialysis probes was also tested by placing the probes CMA20/10 in 50 μ g/ml MT in Ringer's solution and perfusing the fluid at 2 μ l/min. The experiments were done in duplicate sets. All the recovery samples were analyzed by HPLC and the recovery was calculated as:

$$\text{Relative recovery} = \frac{(C_{\text{dialysate}})}{(C_{\text{medium}} - C_{\text{dialysate}})} \quad (1)$$

In vivo recovery of MT by loss was performed by implanting brain microdialysis probes in the olfactory bulb as described in the previous sections. Blank Ringers solution was perfused through the probes during and 120 min after implantation at 2.0 μ l/min. After that, the perfusate was changed to Ringers solution containing 50 μ g/ml of MT and dialysates were collected every 10 min for 60 min. Similarly, the *in vivo* recovery of MT from subcutaneous probes was determined by placing the probes in the subcutaneous tissue and perfusing the probes with Ringer's solution followed by Ringers solution containing MT. The content of MT in the dialysates and in the perfusion syringes was analyzed by HPLC. The experiments were done in duplicate sets. The *in vivo* recovery by loss was calculated as:

$$\text{Relative recovery} = \frac{(C_{\text{perfusate}} - C_{\text{dialysate}})}{(C_{\text{perfusate}})} \quad (2)$$

HPLC assay

The HPLC system consisted of a multi-solvent module 600E pump, 717 plus auto-sampler (Waters Corporation, Milford, MA) an EC detector (ESA Corp., Chelmsford, MA) were used. Coulochem II amperometric detector equipped with a glassy carbon electrode cell and an Ag/AgCl reference electrode was used. Isocratic separation was achieved at 27°C using YMC-ODS-AQ column (150 mm × 3 mm; Waters Corporation, Milford, MA). The working electrode was set at an applied potential of 700 mV relative to an Ag/AgCl reference electrode, filter setting was 0.1 Hz, and range setting was 10 nA. The mobile phase was consisted with 7.2 mM citric acid and 11 mM sodium dihydrogen phosphate as supporting electrolyte in water-methanol (8:2) system. Each run needed 30 min at a flow-rate of 0.5 ml/min. This method provided MT detection limit of 200 pg/ml.

Data analysis

The steady-state flux (J_{ss}) and permeability coefficient (P) across nasal epithelium (EpiAirway™-100) from MT formulations were determined according to the below-given equations (3 and 4):

$$J_{ss} = V/A (dC/dt) \quad (3)$$

$$P = J_{ss} CF^{-1} \quad (4)$$

Where, J_{ss} is the steady-state flux in $\text{mg cm}^{-2} \text{h}^{-1}$, V is the receptor volume in ml, A is the active diffusion area in cm^2 , C is the receptor concentration in mg ml^{-1} , CF^{-1} is the fraction of solubilized drug in the formulation, and t the time. The steady-state flux was determined from the slope of the linear plot of cumulative amount of MT permeated versus time.

In vitro and *in vivo* data were subjected to statistical analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using GraphPad Prism software version 4.0 (San Diego, CA). Probability values of less than 0.05 were considered as statistically significant.

For *in vivo* data, a limited PK analysis was performed using WinNonlin Professional (Version 4.1, Pharsight, Mountain View, CA). The PK parameters calculated were observed maximum plasma concentration ($C_{\max}$), the time to reach the maximum plasma concentration ($T_{\max}$), plasma half-life ($t_{1/2}$) and F-subcutaneous and F-olfactory concentrations of MT.

Results

The DSC and HPLC data of milled MT and untreated MT indicate that the milling process did not cause any alteration in the physical and chemical nature of the drug (data not shown). Formulations of MT with PEG400 and CMC vehicles behaved approximately as Newtonian fluids in the range of shearing rates applied, whereas carbopol exhibited shear-thinning properties (Figure 2). In case of PEG400, there was no apparent change in the viscosity with higher shear rates. Therefore, the shear rates were not studied beyond 50 (1/s). As previously reported, the viscosity plays an important role in the DSD from nasal pumps (Dayal et al., 2004). In general, an increase in the viscosity of a solution makes it more difficult to atomize the liquid into small droplets from nasal pump devices. Liquids with viscosity greater than 40 cP produce larger droplets and make them not suitable for nasal products. Although, carbopol formulation exhibited a higher viscosity under the shear rates compared to CMC, the DSD from the nasal pumps were lower than CMC. The span values,

which are an indication of the width of the distribution, were considerably lower for the carbopol formulation (Table 1).

Solubility, *in vitro* drug release and permeability across nasal mucosa

The solubility of MT in PEG was very high (226.18 ± 9.68 mg/ml) and low in carbopol and CMC (2.11 ± 0.04 and 1.73 ± 0.01 mg/ml, respectively). The *in vitro* drug release profiles of MT formulations are shown in Figure 3. The cumulative % release of MT from carbopol and CMC formulations were higher than PEG400 and PEG400/water (1:1) solution-based formulations. We expected the solution-based formulations to have the faster *in vitro* drug diffusion rates. However, we initially attributed the lower rate due to two factors. First being, that MT partition across the membrane was retarded by the PEG vehicle. Secondly, the high viscosity of PEG retarded MT release due to its affect on the MT diffusion coefficient. This second phenomena was eliminated since the release profile did not change appreciably after the viscosity was reduced by dilution with 50% water for the MT-PEG400 formulations. The effect of formulation on the permeation of MT across EpiAirway™ tissue is presented in Figure 4. The steady-state flux and permeability coefficient data are presented in Table 2. The flux of MT from CMC and carbopol were 1.2 and 1.3 times higher, respectively, as compared to PEG400 solution ($P < 0.01$). The permeability coefficient of MT was 150- and 190-fold higher for carbopol and CMC, respectively, as compared to PEG ($P < 0.001$) indicating high thermodynamic activity of MT in the polymer vehicles. Thermodynamic activity is maximum at the saturation solubility of MT in the vehicles. In the present study, both CMC and carbopol vehicles were presented with MT as suspensions where the polymeric vehicles are saturated with MT, whereas in case of PEG400 the formulation is a clear solution; therefore, MT has affinity to PEG400 rather than partitioning into the tissue. This explains the lower permeability coefficient of PEG400.

In vivo microdialysis studies

The *in vitro* and *in vivo* relative recovery of MT from microdialysis probes is presented in Figure 5. The *in vitro* recoveries of brain and vascular microdialysis probes were 52% and 47%, respectively (Figure 5A). *In vivo* recoveries of MT from brain (olfactory bulb) and vascular microdialysis probes, as determined by retrodialysis, were 18% and 32%, respectively (Figure 5B). Figure 6 depicts the brain concentration-time profiles of the MT from different formulations in the olfactory bulb following intranasal administration. The area under curve (AUC) of MT concentration—time profiles and pharmacokinetic parameters of the nasal formulations are presented in Table 3. The subcutaneous tissue levels for the suspension-based formulations (carbopol, CMC) were higher compared to the high viscosity MT-PEG400 formulation (Figure 7). The AUC of carbopol was approximately 2.5-fold greater compared to MT-PEG400. Similarly, the AUC of carbopol was ~50% lower as compared to IV administration. The uptake of MT into the olfactory bulb was dependent on the formulation; carbopol exhibiting the highest AUC. The levels of MT in the olfactory bulb after intranasal administration from different formulations were 4 to 9-fold higher as compared to IV administration suggesting enhanced delivery of MT via nasal route. The levels of MT in the olfactory bulb rose rapidly and reached their maximum levels in 20 min. However, the levels of MT in the olfactory bulb were barely detectable at 10 min but raised and reached their maximum levels after 20 min for IV administration.

Discussion

Rheological and DSD of MT formulations

In preliminary experiments, selected polymers from cationic, nonionic and anionic categories were screened for suspendability, sedimentation volumes and rheological properties etc. to identify potential MT suspension vehicles (data not presented).

Rheological and DSD studies were conducted to eliminate formulations that were considered nasally undeliverable. For example, several MT formulations using polymers with medium or high viscosity grade polymer were eliminated based on their influence on DSD. Earlier we reported that increases in molecular weight of hydroxypropyl methyl cellulose resulted in a larger DSD while still maintaining the same viscosity of the solutions (Dayal et al., 2004). In addition, formulations with high viscosity that generated median droplet size (Dv_{50}) above 120 μm were also eliminated from the studies. The criteria for selecting formulation with Dv_{50} below 120 μm was based on commercially available nasal products that were previously tested have generated Dv_{50} values in the range of 45–120 μm . Non-aqueous vehicles such as PEG400, propylene glycol can be used in the nasal formulations. Despite its high viscosity and large Dv_{50} value, several reports have shown that these vehicles improve bioavailability by hindering mucociliary clearance. For this study, we selected PEG400 as a control vehicle for MT solution formulation.

The flow properties of a polymer solution result from the polymer conformations in solution. Both inter- and intra-molecular interactions influence the rheological behavior of gel solution (Picout and Ross-Murphy, 2003). At low polymer concentrations, polymers form only small aggregates which behave like single polymer chains. At low polymer concentrations, intra-molecular interactions usually dominate in smaller polymer coils compared with random coils. At high polymer concentrations, however, inter-molecular interactions occur so that clusters are rapidly formed with cross-links between different polymer chains causing growth into a loose three-dimensional structure. The changes in viscosity for the carbopol formulation can be explained by aggregation of the polymer units. The polymer consists of small particles that swell when added to water and exhibits a high degree of cross-linking to build a continuous network. However, upon applying shearing stress, the viscosity decreases and the polymer behaves as an intra aggregates suspended particles. The consequence of shearing force is the decrease in the strength of inter-molecular interactions between polymer chains. This is evident by the breakup of the high yield value and further reduction in viscosity as shearing stress is applied (Figure 3). Nasal droplets are formed by accelerating a liquid with pressure through a turbine vane located inside the nozzle of a nasal pump. The high shear forces generated by acceleration of the liquid tend to break many weaker inter-molecular bonds of polymer solutions resulting in less cohesion and generation of smaller droplets. Thus, shear-thinning behavior of carbopol solutions supports the lower DSD values as compared to CMC formulations from the various nasal pumps (Table 1).

In vitro release and permeability of MT

The relatively lipophilic PEG vehicle retarded MT from partitioning across the membrane. The release rate increased with time for PEG formulations, which is attributed to osmotic actions of the PEG400 vehicle. This osmotic effect from the PEG vehicle caused the dilution of the formulation in the receptor phase resulting in the acceleration of release of MT. This effect was noticed with the increase in the volume in the receptor phase following diffusion studies. The osmotic effects of PEG as well as the lipophilic properties of PEG400 correlate with the low permeation of this formulation. There was an inverse relationship between the solubility and permeability coefficient for the vehicles used in this study. Carbopol showed the highest flux and permeability coefficient for MT. The increase in the above parameters is due to the enhanced solubility of MT in the polymer vehicle. The permeability coefficient of MT with carbopol and CMC were 150 and 200 times higher than PEG400, suggesting high thermodynamic activity of these formulations. PEG400 was a good solubilizer for MT ($226.18 \pm 9.68 \text{ mg ml}^{-1}$), and had a high affinity for MT, resulting in a poor release of MT. The mechanism of enhancement of MT permeability with carbopol or CMC vehicles could not be clearly attributed to changes in the tight junctions or cell integrity. TEER

measurements ranged from approximately 1100 to 750 Ωcm^2 in the buffer during the course of the experiment. No significant reduction in TEER measurements from the formulations were observed compared to buffer solution ($P > 0.05$). MTT assays showed no significant reduction in cell viability compared to control during the permeation experiments ($P > 0.05$). This study demonstrates that suspension vehicles could enhance the permeation of MT although the mode of enhancement may be different for each vehicle. There was no correlation between steady state flux and solubility values thus indicating that high solubility values do not translate to high drug permeation. The present study indicates that CMC and carbopol showed significant MT permeation across EpiAirway™ tissue compared to PEG400 solution and may be used as potential vehicles for the nasal delivery of MT.

In vivo microdialysis studies

The recovery depicts the overall mass transport of a substance to and from the microdialysis probe. The mass transport from the microdialysis probe involves contributions from the analyte diffusion through the dialysate, the membrane, and the tissue extracellular fluid space. Relative recovery is theoretically independent of the compound concentration since the concentration gradient and partition coefficient is proportional to the amount, which diffuses into the perfusate (Kreilgaard, 2002). Stenken et al. (Stenken et al., 1997) observed microdialysis recoveries as high as 70% for phenacetin and antipyrine. The results of the present study indicate good recovery of MT from both concentric (brain) and linear (subcutaneous) microdialysis probes.

The plasma concentration versus time curves of MT indicate that disposition of MT in rat blood has a slower and longer elimination phase. The lack of increased levels of MT in CSF may be due to the uptake of MT into the CNS tissue. A study reported by Dahlin (Dahlin et al., 2000) on the uptake of dopamine in mice brain, showed a concentration gradient of radiolabeled dopamine from the olfactory after intranasal administration. The CSF was not the carrier for dopamine, as dopamine label was not evident in the striatum. Brain delivery of insulin via intranasal administration has been reported as a novel approach to the treatment of neurodegenerative disorders. Insulin receptors are prevalent in the limbic and hippocampal regions and enhanced memory following brain delivery of insulin was reported (Benedict et al., 2004, Benedict et al., 2007). Intranasal delivery of insulin to the brain along olfactory pathways was beneficial in Alzheimer's disease and other brain disorders, without any apparent alterations in the profiles of systemic blood levels of insulin or glucose (Frey and William, 2001). Intranasal insulin dose dependently modulated verbal memory in memory impaired older adults (Reger et al., 2008a) and also modulated β -amyloid in early AD (Reger et al., 2008b). van den Berg et al. (van den Berg et al., 2004) reported that there is no additional uptake of MT in the CSF after nasal delivery compared to IV administration. The formulation and the dose of MT delivered to animals could have shown differences in the results of their study. While lipophilic and highly permeable drugs may be able to distribute from the olfactory mucosa to the central brain, the directly transported fraction would probably be insignificant compared with molecules crossing the blood–brain barrier. For hydrophilic drug molecules absorbed via paracellular transport, the blood–brain barrier may restrict the absorption from the circulating blood. However, the difficulty toward absorption via the olfactory pathway and distribution to the central brain may be too high to achieve a pharmacological response especially for less potent drugs. Following intranasal administration, the drug was generally localized in higher amounts in the olfactory region of the brain. This indicates that the drugs predominantly follow olfactory route of drug delivery to the CNS (Jain et al., 2010, Wang et al., 1998, Yamada et al., 2007). Highly potent drug candidates, or candidates not prohibited by poor aqueous solubility for nasal administration are also substrates for efflux transporters in the blood–brain barrier. The elimination of polar metabolites from the CSF is much slower than the parent compound. Therefore, the

analgesic potency of morphine-6-glucoronide after intracerebroventricular or intrathecal administration in rats is from 45 to 800 times greater than that of morphine (Christrup, 1997) and such compound can be a good candidate for nasal delivery. Following intranasal administration of lidocaine to rats, trigeminally innervated structures (teeth, temporomandibular joint, and masseter muscle) were found to have up to 20-fold higher tissue concentrations of lidocaine than blood (Johnson et al., 2010). Our studies demonstrated that the brain (olfactory bulb) levels of MT after intra nasal administration were 9.22, 6.77 and 4.04 fold higher for carbopol, CMC and PEG400, respectively, than that of intravenous MT in rats. In addition to delivery through the olfactory pathway, trigeminal and other vascular pathways pathway might have contributed to enhanced brain delivery of MT. The trigeminal nerve enters the brain from the respiratory epithelium of the nasal passages through cribriform plate near the olfactory bulbs, creating entry points into both caudal and rostral brain areas following intranasal administration to enhance brain levels of drugs (Dhuria et al., 2010). Our results are in good agreement with reports in the literature, where nasal dosage forms have been shown to give improved drug absorption mainly due to their ability to reside longer in the nasal cavity before being cleared by the mucociliary clearance system. MT concentrations are required in the femtomolar range to bind to various MT receptors in brain (Paul et al., 1999). Various brain binding site concentrations of MT ranged from 8 to 14 fmol/mg protein in bovine brain regions (Cardinali et al., 1979). The average endogenous plasma concentration of MT during day time is low (<10 pg/ml), and at night the MT levels rise to 30–120 pg/ml (Lewy and Newsome, 1986). Thus, the normal brain/pineal gland concentrations of MT were reported in the picogram range. The concentrations achieved our experiments (1–6 nmol) are much high enough to induce pharmacological actions.

In conclusion, suspension formulations for nasal delivery of MT were optimized for DSD, polydispersity and rheological behavior. Microdialysis studies indicate that MT was transported into the CNS via the olfactory bulb following intranasal administration. Nasal administration of MT suspensions in CMC or carbopol increased the brain levels of MT by 6.77- and 9.2-fold, respectively, as compared to intravenous MT solution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Left picture shows: 6-well plate with 6 culture inserts containing EpiAirway™ cell cultures placed in a rotary bath maintained at 37°C. The diagram at right depicts the culture inserts in the culture wells showing tissue at the Air-Liquid Interface. Apical (top) surface of tissue is exposed to air allowing for direct application of test formulation.

Figure 2. Rheological properties of MT formulations under shear rate using LV-DV-III Brookfield viscometer with a small sample adapter.

Figure 3. Effect of formulation on the release of MT through dialysis membrane. Values represent mean $\pm$ SD, $N=6$.

Figure 4. Effect of formulation on the permeation of MT across EpiAirway™ tissue. Values represent mean $\pm$ SD, $N=6$.

Figure 5.
In vitro and *in vivo* recovery of MT determined through concentric brain microdialysis probe (A) and vascular microdialysis probe (B) at a perfusion rate of 2 μ l/min.

Figure 6.

Influence of route of administration on MT concentration in brain as assessed by brain (olfactory) microdialysis sampling in rats. Values represent mean $\pm$ SD, $N=3$.

Figure 7.

Influence of route of administration on MT concentration in subcutaneous tissue as assessed by cutaneous microdialysis in rats. Values represent mean $\pm$ SD, $N=3$.

Table 1

Effect of nasal pump design and formulation vehicles on droplet size distributions.

Nasal Pump	Formulation	Dv ₁₀ (μm)	Dv ₅₀ (μm)	Dv ₉₀ (μm)	Polydispersity
High Viscosity *	Carbopol	36 ± 2	63 ± 3	96 ± 1	1.0 ± 0.7
	CMC	40 ± 2	78 ± 7	188 ± 2	1.9 ± 0.2
	PEG400	65 ± 1	158 ± 5	245 ± 0	1.1 ± 0.0
Upside down *	Carbopol	35 ± 1	62 ± 2	96 ± 1	1.0 ± 0.0
	CMC	36 ± 1	82 ± 4	182 ± 3	1.8 ± 0.1
	PEG400	65 ± 3	164 ± 7	239 ± 2	1.1 ± 0.1
HV90 **	Carbopol	32 ± 2	67 ± 4	99 ± 2	1.0 ± 0.1
	CMC	32 ± 3	88 ± 5	174 ± 6	1.6 ± 0.1
	PEG400	62 ± 1	157 ± 7	235 ± 6	1.1 ± 0.0

* Pfeiffer's high viscosity and upside down design pumps.

** Camlar's HV90 pump representing a spray angle 90°.

Table 2

Effect of formulation on the solubility, flux and permeability coefficient (K_p) of melatonin across Epiarway™ tissue.

Formulation	Solubility (mg.ml ⁻¹)	Flux (mg.cm ⁻² .h ⁻¹)	K_p (cm.h ⁻¹)
PEG400	226.18 ± 9.68	0.1940 ± 0.0012	7.94 ± 1.74*10 ⁻⁴
CMC	1.73 ± 0.01 ***	0.2424 ± 0.0082 **	0.1530 ± 0.028 ***
Carbopol	2.11 ± 0.04 ***	0.2555 ± 0.0764 **	0.1210 ± 0.169 ***

**
 $P < 0.01$ versus MT-PEG400;

 $P < 0.001$ versus MT-PEG400.

Table 3

Effect of formulation and delivery route on the brain (olfactory bulb) and subcutaneous tissue concentrations of MT in rats.

Formulation	AUC-Olfactory bulb (ng·h)	AUC-systemic (ng·h)	F-systemic	F-olfactory
PEG400	27.90 ± 7.58	221.2 ± 35.6	0.19	4.04
CMC	46.55 ± 23.79 **	483.7 ± 265.3 *	0.41	6.77
Carbopol	63.46 ± 10.77 **	580.3 ± 232.9 **	0.49	9.22
MT solution (IV route)	6.88 ± 2.31	1195.8 ± 303.2 **	1.00	1.00

* $P < 0.05$ and

** $P < 0.01$ compared to MT-PEG400.

F-olfactory = (AUC_{nasal}/AUC_{i.v.}); F-systemic = (AUC_{nasal}/AUC_{i.v.}).