REVIEW

Factors involved in the design of nasal delivery systems for peptides and proteins

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ABSTRACT

Because the nasal mucous is one of the most permeable areas, the intranasal delivery route is the most promising for proteins and peptides to reach the central nervous system (CNS). Nevertheless certain aspects of these macromolecules limit their bioavailability, such as molecular weight, the rapid mucociliary clearance mechanism and enzymatic degradation. This paper critically summarizes the factors participating in the design of systems for the nasal delivery of peptides and proteins. The physicochemical properties of the biomolecules and features of the formulation design that directly influence their bioavailablity and stability are presented. Pharmaceutical aspects include an analysis of the influence of excipients that act as stabilizers, antimicrobial preservatives, absorption enhancers, and bioadhesive polymers as well as the administration device. The use of molecules involved in endogenous neuroprotection administered intranasally is a recent proposal in the development of new drugs. Neurotrophic factors, interferon beta-1b, erithropietin (i.e.: rH-EPO, Neuro-EPO) and insulin, are among the biotechnology products administered intranasally. These new products show greater therapeutic potential as putative neuroprotectors in neurological diseases, both for treatment during the acute phase, and for the chronic stage.

Keywords: intranasal delivery, peptides, proteins, excipients, formulation, neuroprotection

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RESUMEN

Factores que intervienen en el diseño de sistemas de liberación nasal de péptidos y proteínas. La vía nasal es una de las rutas más prometedoras para la administración efectiva de péptidos y proteínas hacia el sistema nervioso central, por la permeabilidad de la mucosa nasal. Sin embargo, estas macromoléculas tienen características como el peso molecular, el rápido aclaramiento mucociliar y la degradación enzimática que limitan su biodisponibilidad. Este trabajo resume de forma crítica los factores que intervienen en el diseño de sistemas de liberación nasal de péptidos y proteínas. Se exponen propiedades físico-químicas de las biomoléculas y del diseño de la formulación que influyen en su biodisponibilidad y estabilidad. Se presenta un análisis de la influencia de algunos excipientes que actúan como estabilizantes, preservativos antimicrobianos, promotores de la absorción y polímeros bioadhesivos, así como el dispositivo de administración. La administración intranasal de moléculas involucradas en la neuroprotección endógena es una propuesta reciente en el desarrollo de nuevos neurofármacos. Los factores neurotróficos, el interferón β-1b, la rH-EPO, la Neuro-EPO y la insulina son algunos de los productos biotecnológicos que se administran por esa vía, y suelen poseer elevada potencialidad neuroprotectora durante las fases aguda y crónica de las enfermedades neurológicas.

Palabras clave: administración intranasal, péptidos, proteínas, excipientes, formulación, neuroprotección

*I*ntroduction

The administration of drugs by the nasal route has been used for several years to achieve an effective and non-invasive therapy. Due to its surface, its large vascularization and epithelium characteristics, the nasal mucosa is highly permeable, thereby accelerating the therapeutic effect. It thus initially avoids hepatic metabolism, while favoring the rapid release of the active substance [1, 2].

Hence, the nasal route is a viable and attractive option for the supply of macromolecules such as peptides and proteins. However, some properties lead to the low bioavailability of these compounds (1-3 % systemic absorption) [3, 4] and less than 1 % goes to the central nervous system (CNS) in the absence of absorption enhancers [5].

This route is highly promising for macromolecule administration since the nasal mucosa is a highly permeable surface. Its main drawbacks for drug absorption include the difficult transmembrane transport for macromolecules having a size of over 1 kDa, and the rapid mucociliary clearance (MCC) and enzymatic degradation [2].

During the development of a new drug, strategies are followed to increase bioavailability of peptides and proteins. These cover the inclusion of absorption enhancing excipients, increasing residence time of the drug in the nasal cavity and inhibiting enzymatic degradation. The appropriate selection of excipients is also important to avoid microbiological contamination and ensure the stability of the formulated protein.

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The administration device must be carefully chosen since it affects the therapeutic effectiveness of the drug [6].

In this paper we critically analyze the main biological, physicochemical and pharmaceutical factors affecting the design of peptide and protein formulations for nasal administration. Furthermore, we present some of the biotechnological products that are delivered through that route, and have greater potential as effective protecting agents in neurological diseases.

Anatomical and physiological factors

The nasal cavity has an important protective function by permitting the filtration, heating and humidification of the inhaled air that is conducted to the regions of the respiratory tract (the nasal septum, the respiratory region, the olfactory region and the nasopharynx). The respiratory region is considered to be the most permeable one because it covers a larger area and is highly vascularized. The cellular composition and organization of its epithelial layer is able to maximize the access of air to the structures where neuronal detectors are located [7-10]. In the olfactory region neurons are scattered with olfactory receptors, between the support cells and the basal cells, forming the olfactory epithelium. These cells are bipolar sensorial neurons that mediate the sense of smell and deliver the sensorial information from the peripheral environment to the CNS, through dendrites that are extended throughout the mucosa layer of the olfactory epithelium.

When a drug is administered through the nasal route, a part of the formulation is normally removed through MCC, which takes it towards the nasopharynx, and from there to the gastrointestinal tract, where it is eliminated. Depending on the physicochemical characteristics of the drug and its deposition site, it may follow several routes. There is evidence that the low molecular weight active pharmaceutical ingredients with hydrophobic characteristics have high levels of systemic absorption or they pass to the blood stream at the respiratory region. The hydrophilic macromolecules such as proteins and peptides present low bioavailabilities in the absence of absorption enhancers [3, 9, 11].

After reaching the bloodstream the drugs are distributed in tissues and organs. The macromolecules with a molecular weight of under 600 Da and lipophilic characteristics, may cross the blood-brain barrier and enter the cerebral-spinal fluid (CSF) and tissues of the CNS.

It is stated that macromolecules penetrate into the CSF by two routes: the olfactory route and the trigeminal nerve. The molecules reaching the olphactory epithelium may enter the dendrites of these neurons through pinocytosis, simple diffusion or by endocytosis, mediated by receptors and slowly transported through the axon of the olphactory nerve [5]. If the molecules find discontinuities between the support cells produced by the systematical renovation of this epithelium, there may be a rapid movement towards the CNS through the basal cells and the fissures between the supporting cells and the receptor [9, 12]. Therefore, the barrier at the nasal zone toward the CNS may be favorably permeable through the constant formation of olfactory neurons.

The intranasal application of drugs enables their rapid passage to the systemic blood stream by crossing through the nasal epithelium and penetrating the capillaries of the sub-mucosal tissue. The movement generated by the perivascular pump may promote a rapid distribution of the therapeutic molecules in the brain [5, 13, 14].

Although there is no conventional lymphatic system in the brain, some physiological studies have revealed a significant lymphatic drainage from the brain towards the cervical lymphatic glands, according to the immunological point of view [15]. If the active substance remains in the lymphatic system, it may appear in the systemic bloodstream and contribute to the nasal systemic route [16]. The passage of molecules to the CNS through the nasal route is important and functional, which is evidenced by the fact that they arrive without having entered the blood in large amounts [5, 17].

Barriers limiting the absorption of proteins and peptides

The most important factor limiting the nasal absorption of polar drugs and especially of molecules having high molecular weights such as peptides and proteins is the low permeability of the blood-brain barrier. For the absorption of a drug, it may first cross the respiratory or olfactory nasal epithelium, depending on the deposition site of the formulation. It has been demonstrated that macromolecules may cross the nasal epithelium through passive diffusion or endocytosis, although the amounts are very much restricted and time is relatively long. The cells of the nasal epithelium are interconnected at the apical side through the tight bonds controlling the diffusion of ions and molecules. These zones are dynamic structures that may be regulated and they may open or close depending on extracellular conditions. Because the proteolytic activity is less at the extracellular space compared to the presence of cytosolic enzymes at the intercellular space, it is considered to be the most feasible route for transporting proteins and peptides [18-20].

Another factor is the rapid MCC, which is especially relevant for biomolecules that does not readily cross the nasal epithelium. Liquid and powder nasal formulations that do not include mucoadhesives are cleared by this route in 15 to 20 minutes [1].

A third factor involved at a lower degree is the possibility of enzymatic degradation in the lumen of the nasal epithelium, where the exopeptidases act on the N and C terminal bonds (mono and di-aminopeptidases) and endo-peptidases may act on the internal peptidic bonds [1, 19, 20]. However, the peptidase levels at the nasal epithelium are less than those of the gastrointestinal tract, making this route less susceptible for enzymatic degradation than the oral route [21].

Physicochemical factors

Molecular weight and size, solubility and lipophilic features are determinants in the absorption of therapeutic agents. The bioavailability of drugs with a molecular mass of over 1 kDa may be determined by their molecular weight and by other physicochemical properties. The permeability of drugs with a molecular weight of less than 300 Da is not significantly

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influenced by physicochemical properties because of their greater permeability through aqueous channels in the membrane [22].

Molecular weight

At greater molecular weights there is a lower diffusion coefficient. Molecules having molecular weights of over 1 kDa cross the cell membrane, a process difficult for the others, at least through passive diffusion [23, 24].

The influence of molecular weight on bioavailability of macro-molecules in the absence of absorption enhancers has been evidenced previously (Table 1).

Table 1. Bioavailability of peptides and proteins administered by the nasal route in the absence of absorption enhancers

Peptides and proteins	Molecular weight (Da)	Bioavailability (%)	Model (route)	Reference
Octreotide	991	18.0	Humans (s.c.)	[25]
Salmon calcitonin	3432	3.0	Humans (i.m.)	[26]
Parathyroid hormone (1-34)	4118	2.0	Humans	[27]
Insulin	5808	0.3	Rat	[28]
		0.9	Rabbit	
rhG-CSF	~ 18 800	2.0	Rat	[29]
Recombinant human interferon-αB/D	~ 19 000	2.9	Rabbit	[30]

rhG-CSF: recombinant human granulocyte colony-stimulating factor. s.c.: subcutaneous route. i.m.: intramuscular route.

Hydrophobicity and hydrophilicity

The distribution and number of hydrophobic residues in the biomolecule is a property determining its solubility in a solvent. Hydrophobic groups tend to be clustered within the protein molecules, although many of them remain exposed on the surface and determine their behavior, such as their charge and other polar groups [31, 32].

Proteins are compounds with essentially hydrophilic characteristics; for example, insulin has a reported butanol/water partition coefficient of -1.08 [33]. Several studies describe the effect of lipophilicity in the absorption through the nasal route, but most of the cases have used drugs of low molecular weight and high values of the partition coefficient [34-36]. Sakane *et al*. studied the relationship between lipophilicity and levels transported towards the CSF through the nasal cavity of hydrophilic sulfonamides in phosphate buffer with different octanol/water partition coefficients (0.012, 0.250, 0.261 and 0.892). A significant correlation was observed between the drug concentration in the CSF and the increase in lipophilicity, thereby demonstrating a greater absorption with higher lipophilicity [37].

Physicochemical stability

One of the greatest challenges in the development of liquid protein and peptide formulations is its physicochemical instability, regardless of the administration route. The most common physical instability of proteins is aggregation, which may decrease biological activity, solubility and alter immunogenicity, although this last is not frequently found. However, the presence of any insoluble aggregate in a pharmaceutical protein solution is unacceptable. Protein aggregation may be induced by an array of physical factors such as temperature, ionic force, shaking and superficial/interface adsorption. These factors may increase the hydrophobic surface area of proteins and produce aggregation [38]. It has been demonstrated that the protein aggregation generally increases with concentration due to the rise in inter-molecular interactions [39-41].

On the other hand, proteins are subjected to a variety of chemical modifications because of degradation by deamidation, isomerization, hydrolysis, the rupture of disulfide bonds, beta elimination, succinimidation, deglycosilation and oxidation [42]. The chemical reactions of many amino acid residues in the protein require certain molecular flexibility, and therefore the reaction speed is favored in small peptides or those in the denatured state. The native conformation of the protein protects, prevents or inhibits its potential chemical degradations. Chemical reactions not always affect conformation and bioactivity. This is influenced by the localization and importance of the transformed residue [38, 43].

*P*harmaceutical factors

Pharmaceutical form

Aqueous solutions for nasal administration are the most widely used pharmaceutical forms, in spite of their rapid nasal clearance. Formulations may be oily solutions, suspensions, emulsions, gels, nasal powders and other newer drug release systems such as liposomes, microspheres, and nanoparticles [44-46]. However, reports on emulsions and ointments are limited. Powdered formulations are combined with bio-adhesives and their most important limitation has to do with a greater level of irritation of the nasal mucosa [47-50].

In the literature, gels from chitosan and those derived from polyacrylic acid are frequently described. Their main advantage is that dripping or loss of the drug after administration is reduced and MCC is retarded. An example of this is the nasal insulin gel used as an alternative to its parenteral administration [51-53].

The new drug administration forms, mainly as microcapsules, liposomes, microspheres and nanoparticles isolate the active pharmaceutical ingredient from the environment by introducing it in a polymeric or lipidic matrix for its controlled release. The number of applications are therefore reduced and its bioavailability is increased, by inhibiting the enzymatic activity in the nasal cavity [44, 45]. The microspheres of biodegradable starch are the most widely used nasal releasing systems for insulin, human growth hormone and desmopressin [46].

Liposomes

Liposomes are sub-microscopic vesicles with a central aqueous cavity surrounded by one or several bimolecular lipid lamellas that are separated by aqueous spaces. The major component of liposomes are lipids (phospholipids), which when placed in an aqueous medium at a temperature that is close to that of its transition phase, will spontaneously form these closed vesicular structures. Liposomes are obtained through different methods, leading to the formation

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of vesicles with features depending on the chosen procedure [54].

The vesicular structure of liposomes and their ability to concentrate lipophilic and hydrophilic substances enable their use in pharmaceutical formulations for the controlled release of drugs. This reduces the potential toxicity of certain drugs or potentiates their bioavailability. Depending on the nature (lipidic composition, size, surface charge) of the encapsulated drug and that of components of the final pharmaceutical form, the liposomal formulation may favor the local or systemic distribution and action. Multilamellar liposomes have been obtained from phospholipids and cholesterol using the double emulsion methodology and later coating it with mucoadhesive agents such as chitosan and carbopol, for the controlled release of the recombinant human insulin. In the *in vivo* evaluation of these coated multi-vesicular liposomes applied in rats through the intranasal route, it was shown that they reduced blood glucose levels for a period of up to 72 hours [55, 56].

Nanoparticles

Recently created, nanoparticles are classified as submicroscopic drug release systems (1-1000 nm). They are obtained from natural or synthetic polymers. Depending on the methodology used to obtain them (spray-dried, evaporation of the solvent, phase separation, ionic gelification), the drug may be completely encapsulated or adsorbed in the polymeric system [57].

Obtaining nanoparticles of antigens for vaccination is a steadily growing field since the surface of the nasal mucosa enables the entrance of many pathogens, making it particularly attractive for immunization. Furthermore, the most widely marketed vaccines today are those of proteic antigens and DNA, which are very unstable and require protection from enzymatic degradation. The nasal vehicles for immunization that are most commonly used are the chitosan base and its salts, the poly-L lactic acid, the poly D,L Lacticcoglycolic acid and alginate [58-60].

In a study in mice, the immune response was compared using two formulations, one with nanoparticles loaded with tetanus toxoid prepared with chitosan, and the other with the free antigen. It was found that the nanoparticles generated a greater G immunoglobulin response in the blood [61].

Administration volume

The volume of the substances that may be administered through the nasal cavity is limited because of their anatomy and size. This may be in the range of 50 to 250 µL, and the optimum amount is of about 100 to 150 µL in each nostril. The excess is rapidly drained towards the nasopharynx and eliminated through the gastrointestinal tract [3, 62, 63].

The effect of the concentration and volume of the dose on bioavailability and biological response has been studied in human beings, using e.g., desmopressin. For this, 300 µg of desmopressin were administered in each nostril through a spray, at doses per volumes of 1×50 µL, 2×50 µL and 1×100 µL in each nostril, respectively. The plasmatic levels obtained were of 20 % for two doses of 50 µL, 11 % for one dose of 50 µL and 9 % for one dose of 100 µL. The biological response was significantly greater after the administration of 2×50 µL [64].

Another study with desmopressin showed higher levels of absorption with the dose of 100 µL with a clearance after 240 min, compared to the dose of 200 µL, which showed a clearance at 120 min [62].

In pilot clinical trials with elderly persons a total volume of 400 and 600 µL of insulin was administered, alternating 100 µL of the formulation in each nostril, every 15 minutes, with administration times of 30 and 45 minutes, respectively, giving positive results for the efficacy of the product [65, 66].

pH and buffering capacity

To avoid nasal irritation and infections, the pH of the formulation must be adjusted to values ranging from 4.5 to 6.5. With pH values ranging from 3 to 10 there are no morphological changes in the mucosa, although the frequency of ciliary movement is affected. The pH values outside this range produce irreversible damage to the nasal mucosa [67, 68]. The lysozymes of the nasal secretions which normally destroy bacteria are inactivated under alkaline conditions, making the nasal tissue more susceptible to microbial infection [21].

The type and concentration of the buffer used should also be taken into account. Three buffers (acetate, citrate and phosphate) have been evaluated at 0.07, 0.14 and 0.21 M concentrations, respectively, to assess their effect on the integrity of the nasal mucosa. The most important signs of irritation were obtained with acetate buffer, showing a linear correlation between the damage to the mucosa and the buffer concentration. To select the most appropriate buffer it is important to guarantee the solubility and stability of the drug, but most importantly the integrity of the nasal mucosa [68].

Osmolarity

The slightly hypertonic and isotonic solutions produce minimal damage to the nasal mucosa, while hypotonic solutions cause irreversible damage. Osmolarity is acceptable between 290 and 500 Omol/kg for the supply of drugs through the nasal route, although formulations of about 306 Omol/kg are preferred to ensure the absence of irritation [4, 68, 69]. During the development of nasal formulations the same isotonicity excipients as those designed for parenteral formulations are generally used [68].

Antimicrobial preservatives

Preservatives are commonly found in nasal formulations. Among the preservatives administered through the nasal route *in vitro* and *in vivo* were: benzalkonium chloride, chlorobutanol, paraben, phenylethyl alcohol, and benzoic acid. Those containing mercury have produced irreversible damage to ciliary movement and should not be used [21]. There is no complete correspondence between the results of *in vitro* and *in vivo* studies on the damage produced by antimicrobial agents on the nasal mucosa. *In vivo* studies have demonstrated that the ciliary tissue is protected by the mucus coat, while in the *in vitro* studies there is a direct exposure, without the presence of the MCC mechanism that dilutes the components of the formulation. This feature may explain the differences between

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experimental results published using both trial systems. In general, formulations without preservatives are preferred, since these components are more strongly related to irritation and allergic rhinitis after a prolonged treatment [1].

Protein stabilizers

Stabilizing agents are used to maintain the biological stability of proteins during their production, storage and transportation, which include sugars and glycols, anti-oxidants, surfactants and polymers [38, 42, 63].

Sugars and glycols

The moderately high concentrations of these compounds inhibit protein oxidation, probably due to the elimination of the hydroxyl radical or by the complexation of metal transition ions. Among the sugars and glycols we may find glucose, maltose, mannitol, sorbitol, glycerin, inositol and polyethylene glycol [70].

Antioxidants

The selection of the antioxidant compound in pre-formulation studies of proteins and peptides is difficult because of their possible chemical interaction with amino acids from the lateral chains of the biomolecules. Ascorbic acid is preferred in the presence of traces of metal ions. A chelating agent such as ethylenediaminetetraacetic acid (EDTA) may be effective when binding to contaminating metal traces that promote the formation of free radicals. In the absence of metal ions, cysteine may act as an effective anti-oxidant. To protect proteins in lipidic membranes against oxidation by light, good results have been obtained using alpha-tocopherol [70].

Non-ionic surfactants

Certain surfactants, mainly the non-ionic ones, are used at low concentrations to stabilize proteins by avoiding their aggregation, and subsequently their denaturalization, at the air-liquid or liquid-liquid interfaces. The addition of surfactants reduces surface tension in aqueous solutions and sometimes increases protein solubilization, thus reducing the proportion of denatured proteins at the interface. The non-ionic surfactants are generally less toxic, less hemolytic and less irritating than ionic surfactants [38, 42, 43, 71, 72].

Bioadhesive polymers

Bioadhesion may be defined as the property of a synthetic or biological material to adhere to biological tissues for a long period of time. In the case of mucosal tissues it is known as muco-adhesion, since in the mucosa the bioadhesive is adhered to the mucosa layer [73-76].

Polymeric bioadhesives prolong the retention time in the nasal cavity through this effect and increase the contact time between the protein and the nasal mucosa on counteracting MCC. Hence, the active pharmaceutical ingredient interacts less with mucosal enzymes. The dehydration of the cellular epithelium after the hydration of the polymer may also promote the temporary opening of the tight junctions and potentiate the paracellular absorption of the macromolecules [77]. This alone does not ensure an increase of the expected therapeutic effect, since there are other biological barriers limiting the absorption of proteins and peptides [62, 77, 78]. Furthermore, these excipients increase the viscosity of the protein formulations and decrease the interactions between molecules, which may favorably affect the inhibition of the aggregation and the increase of its stability [38].

The ideal bioadhesive polymer for drug release systems should have the following characteristics [76]:

- The polymer and its degradation product should not be toxic or absorbable.
- They should not be irritating.
- They should preferably form a tight non-covalent bond with the mucus or the surface of the cellular epithelium.
- Adherence to the humid tissue should be fast and situated at specific sites.
- The active pharmaceutical ingredient must be readily incorporated and there should be no obstacle for its release.
- The pharmaceutical form should not decompose during storage and during its half-life.
- The polymer should be fairly inexpensive so that the pharmaceutical form obtained may be competitive.

The bioadhesive polymers studied in nasal administrations include cellulose derivatives such as: methyl cellulose; hydroxi-propyl-celullose; hydroxi-prophyl-methyl-cellulose (HPMC; for example HPMC K4M) and sodium carboxi-methyl-cellulose [77, 79, 80]; derivatives of polyacrylic acid (polycarbophil, carbomer 934P, 971P and 974P) [77, 51, 81, 82]; chitosan base and its salts (ChiSys™) [83, 84]; pectins with a low degree of methylation (LM-5 and LM-12) [53]; pectin (PecSys™) [53, 85] and dextrans (dextran 40 and 70) [86].

Absorption enhancers

Considering the low bioavailability of proteins and peptides, excipients acting as absorption enhancers are required to ensure the permeability of the nasal mucosa with these hydrophilic macromolecules, without producing damage.

Absorption enhancers should be:

- Of immediate action in one direction with a lasting specific, predictable and adequate effect.
- Immediately after being removed from the epithelial tissue they should recover their barrier properties.
- They should not have systemic and toxic effects.
- They should not irritate or damage the surface of the membrane on which they have been applied.
- They must be physically compatible with a wide range of drugs and pharmaceutical excipients [87, 88].

Table 2 shows some of the absorption enhancers that have been used through the nasal route in preclinical and clinical trials, as well as the proposed mechanisms of action. Although a large array of them have been studied, only a few of them have been shown to be effective and safe for their sustained use in human beings [93].

Table 3 presents some bioavailability studies of hydrophilic macromolecules administered in the presence of absorption enhancers.

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Table 2. Types of absorption enhancers through the nasal route that are most widely used

Type of absorption enhancer	Example	Possible action mechanism	Reference
Biliary salts and their derivatives	Sodium deoxycholate, sodium glycolate, sodium taurodihydrofusidate	Membrane rupture, opening of tight junctions, enzymatic inhibition and mucolytic activity	[89, 90]
Surfactants	Sodium lauryl-sulfate, polyoxy-ethylene-9-lauryl ether and saponins	Membrane rupture	[91, 92]
Surfactant of natural origin	Cyclopenta decalactone (CPE-215®)	Increases fluidity of the membrane, favoring transcellular absorption	[93]
Polyenthylene glycol fatty acid hydroxy-ester	CriticalSorb™	Transcellular absorption potential Action mechanism under study	[93]
Alkyl saccharide	Intravail®	Trans-mucosal absorption potential Action mechanism under study	[93]
Chelating agents	EDTA and salicylates	Complexing calcium ions of the epithelial surface, favoring the opening of tight junctions	[18, 87]
Enzymatic inhibitor	Bestatin, Amastatin, Puromycin	Enzymatic inhibitor	[89, 91]
Cyclic oligosaccharides	α-,β- and γ- cyclodextrin, methyl β-cyclodextrin	Membrane rupture and opening of tight junctions	[94, 95]
Mucolytic agents	N-acetyl cysteine	Decrease of the mucous layer	[50, 96]
Polycationic compounds	Poly-L-arginine, poly-L-lysin, chitosan and its derivatives	The interaction of these cationic compounds with the negatively charged sites at the cell surface, reduces the transepithelial resistance and favors the opening of the tight junctions	[18, 97, 98]
Derivatives of poly-acrylic acid	Polycarbophil and carbopol 934P, 971P and 974P	The formation of poly complexes (acrylic acid) $-Ca^{2+}$ decreases endogenous calcium of the cellular epithelium, which favors the opening of the tight junctions reversibly Enzymatic inhibition	[18]
Tight junction modulator peptide	PN159	Reduces the transepithelial electric resistance and thus favors the opening of tight junctions	[99]

Administration devices

The form of administration and the device used may also affect the absorption of the drug. Nose drops are the simplest dosage system, but their drawback is that the exact dose administered cannot be measured accurately, sometimes producing therefore an overdose. Also, in the absence of muco-adhesives, the drug is rapidly drained from the nasal cavity. The nasal spraying devices can ensure a more accurate dose of the drug since they have dose-metering valves. It is generally considered that the spraying devices apply the drug normally at the atrium, or anterior region of the respiratory epithelium (inferior and medium turbinate). This zone is covered mainly by squamous epithelium, which is normally non-ciliary, and the drugs are eliminated more slowly than the drops. In general, drops are deposited in the upper areas of the respiratory region and the olfactory region, where the epithelium is mainly ciliary and there is a greater incidence of clearance [10, 105, 106].

Table 3. Bioavailability of hydrophilic macromolecules administered by the nasal route in the presence of absorption enhancers

Hydrophilic macromolecules	Molecular weight (Da)	Bioavailability (%)	Model (route)	Absorption promoter	Reference
Salmon calcitonin	3432	27	Rat (i.v.)	N-acetyl-L-cystein	[50]
Insulin	5808	3.4-5.0	Humans (i.v.)	Cyclodextrins	[89]
		7		Chitosan 0.5 %	
		12	Humans (s.c.)	CPE-215®	[100]
rhG-CSF	18 800	8.4	Sheep (s.c.)	SSMS and LPG	[101]
Human growth hormone	22 000	7.8 (0.05 IU/kg)	Humans (i.v.)	L-alpha-phosphatidyl-choline	[102]
		8.9 (0.10 IU/kg)			
		3.8 (0.20 IU/kg)			
		17.4	Rat (s.c.)	5.0, 7.5 and 10 % CriticalSorb™	[93]
		34.4			
		49.9			
Neuro-EPO	34 000	0.26 (CSF)	*Macaca fascicularis*	Polysorbate 80 and EDTA	[103]
FITC-dextran	71 200	0.05	Rats	Sodium taurocholate and EDTA	[104]

CSF: cerebrospinal fluid; DIT: Dextran marking with di-iodine-L-tyrosine; FITC: Fluorescein isothiocyanate; LPG: L-alpha-lysophosphatidyl-glycerol; Neuro-EPO: erithropoietin with low sialic acid content; SSMS: small starch microspheres; rhG-CSF: recombinant human granulocyte colony-stimulating factor; i.m.: intramuscular route; i.v.: intravenous route. s.c.: subcutaneous route; IU: international units.

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In a study using nasal spray and drops to assess the distribution and clearance of formulations with bioadhesive polymers, at the olphactory epithelium region in human beings, higher deposition levels were obtained with nose drops than nasal spray. However, it was stated that the administration method at the supine position with the head leaning backward is important to ensure the absorption of the drug at this epithelium and its delivery to the CNS [53].

New administration devices are now being designed and developed to ensure that the drug reaches the upper regions of the nasal cavity. An example of this is the electronic spray ViaNase™, produced recently be Kurve Technologies, Inc. [107].

Neuroprotective agents supplied by the nasal route in preclinical and clinical trials

Neuroprotection is a therapeutic and profilactic treatment strategy to prevent or countervail neuronal loss by CNS diseases of different origins, such as cerebral infarction, neuro-trauma and neuro-inflammatory and neuro-degenerative diseases. One of the main difficulties in the development of a neurological drug is to have it reach the CNS after passing through the blood-brain barrier. Generally the neuroprotective drugs must be administered into the brain ventricle and parenterally [108].

No sufficiently effective, specific and safe drug for its access to the CNS has yet been found that may be used as a neuroprotector in neurological diseases during the acute and chronic stages. Most of the effective neuroprotective therapeutic agents in ischemia biomodels have failed because they have not been well tolerated clinically [109].

The scientific literature frequently describes the use of the nasal route to deliver drugs to the CNS. This may be explained, among other reasons because of the development of the biotechnology industry, a strong source of peptide and protein drugs. These macromolecules that are generally obtained by recombinant DNA technology or chemical synthesis do not reach the CNS from the bloodstream, because of the fine regulation of the passage of molecules and ions through the blood-brain barrier. However, some macromolecules can cross the blood-brain barrier through two routes that lead directly to the cerebrospinal fluid: the olfactory route and the trigeminal route.

The use of molecules with therapeutic activity, produced by the human body and administered through the nasal route is a recent proposal in neuroscience research. Neurotrophic factors cytokines and insulin are some of these proteins.

High molecular weight proteins have been supplied in some preclinical studies with rodents, such as the nerve growth factor (NGF) of 26.5 kDa, the brain-derived neurotrophic factor (BDNF) of 26.984 kDa and the ciliary neurotrophic factor (CNTF) of 22.706 kDa [110], the fibroblast growth factor (FGF-2) of 24 kDa and the insulin growth factor type 1 (IGF-1) of 7.5 kDa through the intranasal route and their arrival at the CNS has been demonstrated [111], where they are widely expressed. Thorough studies are taking place related to the properties of all these factors in restoring brain tissue by potentiating angiogenesis and axonal regeneration, which is important in the rehabilitation from neuro-degenerating diseases such as cerebral infarct and Alzheimer and Parkinson diseases [112, 113].

Cytokines have been evaluated in other preclinical trials, such as interferon β-1b and recombinant human erythropoietin (rHu-EPO). The former has anti-inflammatory properties and is used intramuscularly and subcutaneously in multiple sclerosis treatment; a chronic disease characterized by the inflammation and demyelination of plates of the brainstem, the cerebellum, the optical nerve and the spinal cord. With the intra-nasal administration of interferon β h-1b a larger amount is delivered at the CNS compared to the intravenous administration [114].

In the case of rHu-EPO, it is a glycoprotein expressed in the brain and regulated by the hypoxia inducible factor-1 (HIF-1). Its receptor (EPO-R) increases its expression during ischemia, suggesting its participation in an endogenous neuro-protective system in mammal brains [115, 116]. The drug was administered in a focal ischemia model in rats where its neuroprotective effects were shown [117]. The potential complication of the treatment with repeated dosages of rHu-EPO was given through the increase of hematopoietic activity, leading to the search for non-hematopoietic variants that would maintain neuroprotective properties [116, 118].

A promising variant in Cuba is the human recombinant erythropoietin with a low sialic acid content (Neuro-EPO), having a sialic acid content of less than 10 molecules per molecule of erythropoietin. At the Center for Drug Research and Development (Cidem), a Neuro-EPO formulation was developed for nasal administration using HPMC K4M as the bioadhesive polymer and disodic salt EDTA as the absorption promoter. This formulation was tested in pre-clinical trials in non-human primates and it was rapidly and safely delivered at the CNS, without stimulating erythropoiesis [104, 119, 120]. In acute treatments in several brain ischemia models in rodents it has shown therapeutic efficacy [116, 121-123]. At the Center of Molecular Immunology (CIM), in cooperation with the National Center for the Production of Laboratory Animals (Cenpalab) and Cidem, work is under way to start phase I clinical trials of this formulation [124], after its recent approval by the State Center for Drug Control (Cecmed).

Regarding testing in human beings, one of the most advanced studies was carried out with insulin [17]. It was shown that the protein passed from the nasal mucosa to the CSF. Pilot studies have demonstrated that it improves memory, concentration and the functional state of patients at early stages of Alzheimer disease without producing alterations in insulin or glucose blood levels [65, 125, 126].

Conclusions

All factors affecting the bioavailability of peptide and protein nasal formulations for their delivery at the CNS must be known at the start of their development since frequently the failure of the formulation is because only the stability period of the product has been considered. Knowing all barriers inhibiting absorption,

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factors influencing their low bioavailability, their therapeutic use and the physicochemical characteristics of the protein studies are necessary pharmaceutical tools for the selection and optimization of the most appropriate excipients in each case.

The development of finished forms using peptides and proteins with therapeutic potentials as neuroprotectors is a current challenge for the international scientific community. The neurotrophic factors, interferon β-1b, the rHu-EPO, Neuro-EPO and insulin are included as possible candidates for the evaluation of new nasal biotechnological products with neuroprotective effects that may produce treatments with better efficacy and safety.

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