International Journal of Pharmaceutics

Flurbiprofen Sodium Microparticles and Soft Pellets for Nose-to-Brain Delivery: Serum and Brain Pharmacokinetics in Rats after Nasal Insufflation

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Section/Category:	
Keywords:	Alzheimer's disease; flurbiprofen sodium; nasal powder; nose-to-brain transport; microparticle; soft pellet
Corresponding Author:	Gaia Colombo, Ph.D. University of Ferrara Department of Life Sciences and Biotechnology: Universita degli Studi di Ferrara Dipartimento di Scienze della Vita e Biotecnologie Ferrara, FE ITALY
First Author:	Laura Tiozzo Fasiolo, Ph.D.
Order of Authors:	Laura Tiozzo Fasiolo, Ph.D.
	Michele Dario Manniello, Ph.D.
	Sabrina Banella, Pharm.D.
	Laura Napoli, Pharm.D.
	Fabrizio Bortolotti, Pharm.D.
	Eride Quarta, Pharm.D.
	Paolo Colombo, Pharm.D.
	Evangelos Balafas
	Nikolaos Kostomitsopoulos
	Dimitrios M. Rekkas
	Georgia Valsami
	Paraskevi Papakyriakopoulou
	Gaia Colombo, Ph.D.
	Paola Russo, Ph.D.
Abstract:	Neuroinflammation in Alzheimer's disease (AD) revamped the role of a preventive therapeutic action of non steroidal anti-inflammatory drugs; flurbiprofen could delay AD onset, provided its access to brain is enhanced and systemic exposure limited. Nasal administration could enable direct drug access to central nervous system (CNS) via nose-to-brain transport. Here, we investigated the insufflation, deposition, dissolution, transmucosal permeation, and in vivo transport to rat brain of flurbiprofen from nasal powders combined in an active device. Flurbiprofen sodium spray-dried microparticles as such, or soft pellets obtained by agglomeration of drug microparticles with excipients, were intranasally administered to rats by the pre-metered insufflator device. Blood and brain were collected to measure flurbiprofen levels. Excipient presence in soft pellets lowered the metered drug dose to insufflate. Nevertheless, efficiency of powder delivery by the device, measured as emitted fraction, was superior with soft pellets than microparticles, due to their coarse size. Both nasal powders resulted into rapid flurbiprofen absorption. Absolute bioavailability was 33% and 58% for microparticles and pellets, respectively. Compared to intravenous flurbiprofen, the microparticles were more efficient than soft pellets at enhancing direct drug transport to CNS. Direct Transport Percentage index evidenced that more than 60% of the intranasal dose reached the brain via direct nose-to-brain transport for both powders. Moreover, remarkable drug concentrations were measured in the olfactory bulb after microparticle delivery. Bulb connection with the entorhinal

	cortex, from where AD initiates, makes promising flurbiprofen sodium administration as nasal powder.
Suggested Reviewers:	Abu Serajuddin serajuda@stjohns.edu
	Alessandro Dalpiaz dla@unife.it
	Daisuke Inoue d-inoue@fc.ritsumei.ac.jp
	Rita Ambrus arita@pharm.u-szeged.hu

Research Article

Flurbiprofen Sodium Microparticles and Soft Pellets for Nose-to-Brain Delivery: Serum and Brain Pharmacokinetics in Rats after Nasal Insufflation

Laura Tiozzo Fasiolo[a,b,§,†], Michele Dario Manniello[c,†], Sabrina Banella[a], Laura Napoli[a], Fabrizio Bortolotti[a], Eride Quarta[b], Paolo Colombo[b,d], Evangelos Balafas[e], Nikolaos Kostomitsopoulos[e], Dimitrios M. Rekkas[f], Georgia Valsami[f], Paraskevi Papakyriakopoulou[f], Gaia Colombo[a,*], Paola Russo[c]

[a]*Department of Life Sciences and Biotechnology, University of Ferrara, 44121 Ferrara, Italy*
[b]*Department of Food and Drug, University of Parma, 43124 Parma, Italy*
[c]*Department of Pharmacy, University of Salerno, 84084 Fisciano (SA), Italy*
[d]*PlumeStars Srl, 43125 Parma, Italy*
[e]*Biomedical Research Foundation of the Academy of Athens, Athens 115 27, Greece*
[f]*Department of Pharmacy, National and Kapodistrian University of Athens, Zographou 157 71, Athens, Greece*

[§]Present address
Centro Regionale Specializzato per i Biomarcatori Diagnostici, Prognostici, Predittivi (CRIBT) - UOC Medicina di Laboratorio
Ospedale SS. Giovanni e Paolo- Azienda ULSS3 Serenissima
Castello 6777, 30122 Venezia, Italy

[†]These authors contributed equally to this work.

***Corresponding Author**:
Prof. Gaia Colombo, PhD
Department of Life Sciences and Biotechnology
University of Ferrara
Via Fossato di Mortara 17/19
44121 Ferrara (Italy)
Tel. +39 0532 455909
E-mail: clmgai@unife.it
ORCID: 0000-0001-9033-2305

ABSTRACT

Neuroinflammation in Alzheimer's disease (AD) revamped the role of a preventive therapeutic action of non steroidal anti-inflammatory drugs; flurbiprofen could delay AD onset, provided its access to brain is enhanced and systemic exposure limited. Nasal administration could enable direct drug access to central nervous system (CNS) via nose-to-brain transport. Here, we investigated the insufflation, deposition, dissolution, transmucosal permeation, and *in vivo* transport to rat brain of flurbiprofen from nasal powders combined in an active device.

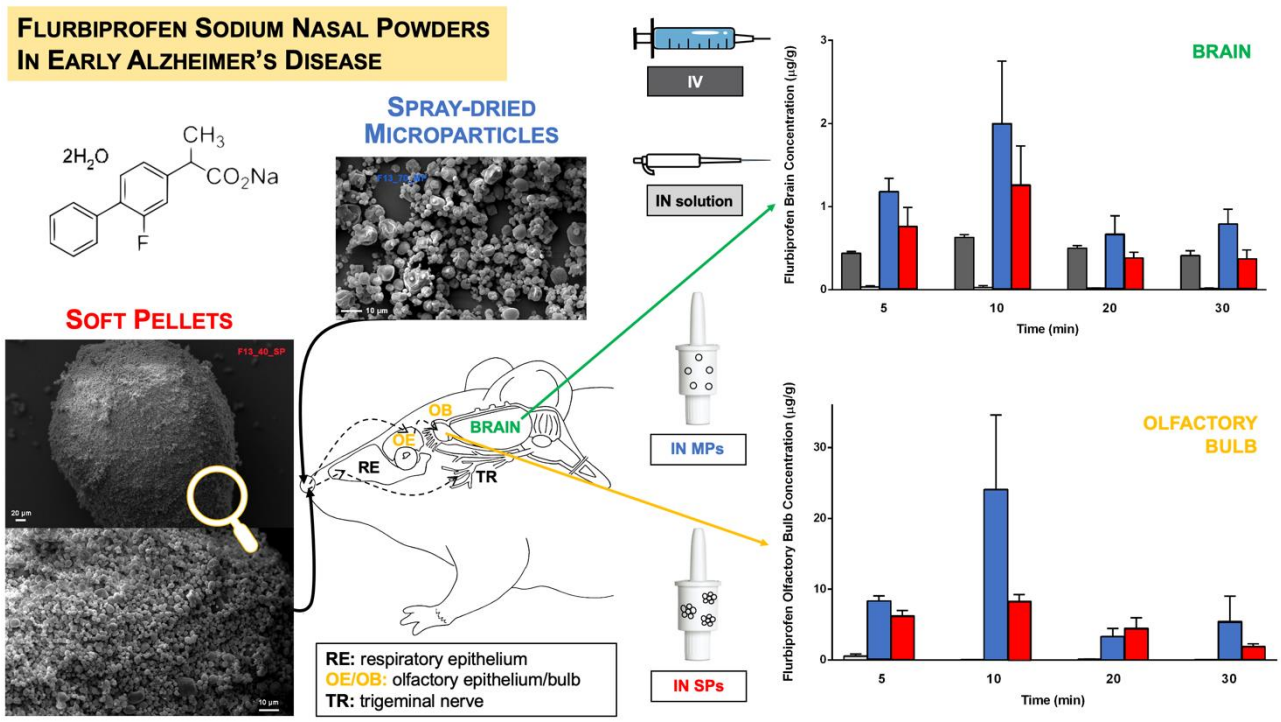
Flurbiprofen sodium spray-dried microparticles as such, or soft pellets obtained by agglomeration of drug microparticles with excipients, were intranasally administered to rats by the pre-metered insufflator device. Blood and brain were collected to measure flurbiprofen levels.

Excipient presence in soft pellets lowered the metered drug dose to insufflate. Nevertheless, efficiency of powder delivery by the device, measured as emitted fraction, was superior with soft pellets than microparticles, due to their coarse size. Both nasal powders resulted into rapid flurbiprofen absorption. Absolute bioavailability was 33% and 58% for microparticles and pellets, respectively. Compared to intravenous flurbiprofen, the microparticles were more efficient than soft pellets at enhancing direct drug transport to CNS. Direct Transport Percentage index evidenced that more than 60% of the intranasal dose reached the brain via direct nose-to-brain transport for both powders. Moreover, remarkable drug concentrations were measured in the olfactory bulb after microparticle delivery. Bulb connection with the entorhinal cortex, from where AD initiates, makes promising flurbiprofen sodium administration as nasal powder.

KEYWORDS

Alzheimer's disease, flurbiprofen sodium, nasal powder, nose-to-brain transport, microparticle, soft pellet.

GRAPHICAL ABSTRACT



LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AUC	Area Under the Curve
BBB	Blood Brain Barrier
C_{max}	Maximum Concentration (peak)
CNS	Central Nervous System
DTE	Drug Targeting Efficiency
DTP	Direct Transport Percentage
FB-COOH	Flurbiprofen
FB-COONa	Flurbiprofen sodium
FDA	Food and Drug Administration
FLD	Fluorescence Detection
HPLC	High-performance liquid chromatography
IN	Intranasal
IV	Intravenous
LOD	Limit of Detection
LOQ	Limit of Quantification
MP	Microparticle/s
MW	Molecular Weight
NCA	Non-Compartmental Analysis
NSAIDs	Non Steroidal Anti-Inflammatory Drugs
PBS	Phosphate Buffered Saline
PK	Pharmacokinetics
RSD	Relative Standard Deviation
SEM	Standard Error of the Mean
SP	Soft Pellet/s
t_{max}	Time-to-peak of maximum concentration
UDS	Unidose Powder System

1. INTRODUCTION

A number of epidemiologic studies identified a link between the long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) and the progression of Alzheimer's disease (AD) in humans (Ali et al., 2019; in t' Veld et al., 2001; Jaturapatporn et al., 2012; McGeer et al., 1996; McGeer et al., 2018). In an animal model, neuroprotective effects have been ascribed to non-selective acidic NSAIDs, including ibuprofen, flurbiprofen or indomethacin (Eriksen et al., 2003). Such action was correlated with their brain uptake via the blood-brain-barrier (BBB) (Parepally et al., 2006). However, the plasma protein binding of acidic NSAIDs limited their brain uptake. For these poorly brain distributed NSAIDs, an improved brain delivery has to be envisaged, since it may promote their activity in the central nervous system (CNS) and reduce the peripheral toxicity (Parepally et al., 2006).

Recently, the role of inflammation in the AD pathology has been focused, postulating the existence of an early and a late inflammation in the CNS (Cuello, 2017). The early neuroinflammation revamped the role of a preventive therapeutic action for AD, that may be more effective than treating the late inflammation phase (Deardorff and Grossberg, 2017; McGeer et al., 2016). Therefore, the anti-inflammatory activity of flurbiprofen is worth being exploited to delay the onset of the disease, provided that its access to brain is enhanced, while limiting systemic exposure (Hershey and Lipton, 2019; Rivers-Auty et al., 2020).

For a drug poorly crossing the BBB after systemic delivery like flurbiprofen (Parepally et al., 2006), the nasal administration could provide a direct access for the drug to CNS. This occurs via transport along the olfactory and trigeminal nerve branches, which innervate the olfactory and respiratory epithelia, respectively (Lochhead and Thorne, 2012). The connection between AD pathology and nose-to-brain delivery of anti-AD drugs is further substantiated by the recently evidenced correlation between alterations of the olfactory nerve and dementia development (Bathini et al., 2019).

In particular, drug transport to brain across the nasal epithelium could be further improved by using nasal powders (Ambrus et al., 2020; Rassu et al., 2018; Tanaka et al., 2016). The solid particle dissolution and drug release in the fluid lining the nasal epithelium sustain the drug passive diffusion rate, owing to the saturation concentration in contact with the tissue (Colombo et al., 2016; Giuliani et al., 2018; Pozzoli et al., 2017).

In a previous work by our group, nasal powders of flurbiprofen sodium, constructed by spray drying, were studied and the *in vitro* dissolution and *ex vivo* transport through rabbit nasal mucosa assessed (Tiozzo Fasiolo et al., 2019). Rapid dissolution rate and fast *ex*

vivo transmucosal transport were obtained with the use of flurbiprofen sodium salt microparticles. Racemic flurbiprofen was chosen, since both enantiomers are of interest in the early AD prevention (Meister et al., 2013). In fact, the S-enantiomer has anti-inflammatory activity, whereas the R one inhibits the gamma secretase enzyme, involved in amyloid plaques deposition (Eriksen et al., 2003; Wong and Ho, 2018).

The aim of the present study was to investigate the insufflation, deposition, dissolution, transmucosal permeation, and *in vivo* transport to rat brain of flurbiprofen sodium nasal powders combined in an active delivery device. To gain an advantage in powder metering and deposition into the nasal cavity, agglomerated microparticles in the form of soft pellets were studied in comparison with the primary microparticulate powders. Thus, two nasal powders were tested, i.e., spray-dried flurbiprofen sodium microparticles and soft pellets thereof. Following insufflation of the powders into rat nasal cavity, flurbiprofen fraction absorbed and brain disposition were assessed, determining drug concentration in serum, olfactory bulb and total brain. For comparison, intravenous and intranasal solutions of flurbiprofen sodium were administered as well.

2. MATERIALS AND METHODS

2.1 Materials

Flurbiprofen raw material (FB-COOH; batch n° T17121044) was kindly donated by Recordati S.p.A. (IT-Milano) and used to manufacture the nasal powders and as high-performance liquid chromatography (HPLC) analytical reference standard. Ibuprofen (batch n° 1301320) obtained from Dipharma srl (IT-Tomba, UD), was used as internal standard. Mannitol (Ph. Eur.) was supplied by Lisapharma S.p.A. (IT-Erba, CO) and lecithin (Lipoid® S45) by Lipoid AG (CH-Steinhausen). HPLC-grade acetonitrile, isopropyl alcohol and methanol were purchased by Merck KGaA (DE-Darmstadt). All other reagents and solvents were analytical grade. A lyophilized flurbiprofen sodium powder was prepared by freeze-drying an aqueous solution of flurbiprofen with NaOH 1M at 7.4 (approx. 2% w/v as flurbiprofen acid).

2.2 Preparation of Flurbiprofen Formulations

2.2.1 Drug solution for intravenous and nasal administration

The drug solution was prepared by adding an excess amount of the lyophilized flurbiprofen sodium powder into water for injection. The suspension was magnetically stirred for 24 h at

room temperature, then filtered through regenerated cellulose membranes (0.45 µm porosity) to collect the clear saturated solution. The flurbiprofen concentration in this solution was equal to 15.61 ± 0.01 mg/ml (Tiozzo Fasiolo et al., 2019). The solution was portioned in 2-ml aliquots into microtubes and stored at -20 °C until use in the *in vivo* experiments. In these storage conditions and as confirmed by HPLC assay prior to use, the solution remained stable.

2.2.2 Spray-dried microparticle powder

The flurbiprofen microparticulate powders for nasal administration were prepared according to our previous publication (Tiozzo Fasiolo et al., 2019). Briefly, a flurbiprofen sodium solution was spray-dried with the Nano spray dryer (B-90, Büchi, CH-Flawil). The liquid feed was prepared by adding NaOH 1M to a flurbiprofen suspension in water (2% w/v), until the drug was fully dissolved (final pH 7.40 ± 0.01). Spray drying conditions were as follows: liquid feed flow rate 1.5 ml/min, relative spray rate 100%, spray nozzle 7.0 µm, inlet temperature 70 °C (batch code: F13_70_MP) or 40 °C (batch code: F13_40_MP). The respective outlet temperatures were 33-34 °C and 29-30 °C. The drug microparticles produced in these conditions contained flurbiprofen sodium salt dihydrate.

2.2.3 Agglomerated powder of spray-dried microparticles (soft pellets)

Since the sodium flurbiprofen microparticles manufactured with the Nano-spray dryer did not spontaneously agglomerate, the soft pellets were prepared according to Balducci et al. (2013). For the purpose, spray-dried excipient microparticles of mannitol and lecithin (ratio 92:8 w/w) were prepared by spray drying a 2% (w/v) total solid solution in water:isopropanol (92:8 v/v) using the Nano B-90 spray dryer (Büchi, CH-Flawil) at an inlet temperature of 40 °C.

The soft pellets were then prepared as follows: spray-dried drug microparticles were manually and carefully mixed with spray-dried mannitol/lecithin microparticles (mass ratio 1:1). After assessing its homogeneous drug content, the microparticle blend was tumbled in a 100 ml glass pan having deflected walls (DISA, IT-Sesto San Giovanni, MI). The pan was fixed to the rotating arm of tablet friability tester at a 90° angle and rotated at 25 rpm/min for 40 min. The agglomerated powder obtained was manually sieved through a 500 µm sieve and collected on top of a 106 µm sieve. Thus, the soft pellets used for *in vivo* administration had a size in the range 106-500 µm.

2.3 Powder Dissolution and *Ex Vivo* Permeation

In vitro dissolution and *ex vivo* permeation of flurbiprofen from the nasal powders were determined with Franz-type vertical diffusion cells (0.58 cm^2), using either a regenerated cellulose membrane or freshly excised rabbit nasal mucosa as barrier. The nasal tissue was extracted within 2 h from the animal's death from rabbit heads supplied by a local slaughterhouse (Pola S.r.l., IT- Finale Emilia, MO). Equipment and experimental conditions were according to our previous research work (Tiozzo Fasiolo et al., 2019). In order to have the same drug amount with both formulations, the powder mass loaded into the cell's donor chamber was about 5 mg for the drug microparticles and 11-12 mg for the soft pellets. The volume of liquid (Phosphate Buffered Saline, PBS pH 7.4; KCl 0.2 g/l; NaCl 8 g/l; Na_2HPO_4 1.15 g/l; KH_2PO_4 0.2 g/l) added to wet the powder was the 100 µl, independently of the powder mass.

2.4 *In Vivo* Animal Experiments

2.4.1 Nasal administration

For the powder administration, a pre-metered single-dose powder insufflator device was employed, i.e., the Unidose Powder System (UDS; Aptar, FR-Louveciennes). The device comprises a mechanical pump connected to a nasal adapter (with a special tip designed for small animals), which includes the reservoir for the solid formulation. Prior to administration, the insufflator's reservoir was filled with about 15 mg of powder accurately weighed, then the device was assembled according to the manufacturer directions. Each loaded device was weighed before and after actuation to determine the quantity of powder administered.

For the intranasal administration of the drug solution, 20 µl were instilled in the rat's nose using a semiautomatic pipette.

2.4.2 Animals and housing conditions

All animal experiments were performed in the animal facility of the Centre of Clinical, Experimental Surgery and Translational Research of the Biomedical Research Foundation of the Academy of Athens. The facility is registered as "breeding" and "experimental" facility according to the Greek Presidential Decree 56/2013, which harmonizes national legislation with the European Community Directive 2010/63 on the Protection of Animals used for Experimental and Other Scientific Purposes. Wistar-type rats were used in the study and were housed in individually ventilated cages (Techniplast, IT-Varese) under

specific pathogen-free conditions and constant environmental conditions (12:12 h light:dark cycle, temperature 22 ± 2 °C, relative humidity 45 ± 10%). The rats were fed on irradiated pellets (2918 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Indianapolis, IN, USA) and had access to tap water *ad libitum*. The cage bedding comprised corncob granules (REHOFIX®, J. Rettenmaier & Söhne Co., DE-Rosenberg). Cages and bedding were changed once-a-week. All rats in the facility were screened regularly according to a health-monitoring program, complying with the Federation of European Laboratory Animal Science Associations' recommendations. The experimental protocol of the study was approved by the Veterinary Authorities of Region of Athens, Greece (Ref. Num. 5043/21-09-2017, EL25BIO03).

2.4.3 Pharmacokinetic study protocol

Forty-eight 8-week-old Wistar-type rats (350 ± 50 g) were randomly divided in four groups. The animals in each group received a different treatment, namely: a) **IV group** (12 rats) received 0.3 ml of the 15 mg/ml drug solution intravenously as bolus through the tail artery (FB-COOH dose: 4.5 mg); b) **IN solution group** (12 rats) received 0.02 ml of the 15 mg/ml drug solution (FB-COOH dose: 0.3 mg); c) **IN microparticle powder group** (12 rats) received intranasally an FB-COOH dose of 6.7 mg as spray-dried microparticles (coded F13_70_MP); and d) **IN soft pellet powder group** (12 rats) received intranasally an FB-COOH dose of 4.2 mg as soft pellets, obtained by agglomeration of flurbiprofen sodium microparticles with mannitol-lecithin excipient microparticles (code F13_40_SP).
All treatments were carried out on anaesthetized rats. Anesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (0.1 mg/kg). The intranasal administration procedure was different for the solution and powders. In the first case, the animals lay in supine position and 5 μl fractions up to 20 μl were instilled alternately into both rat's nostrils, thus aiming to avoid nasopharynx deposition and respiratory distress. The administration time was less than 1 min. For powder administration, the rat lay down on the right side, making the left nostril accessible. Only the left nasal cavity was used for powder insufflation. The tip of the nasal insufflator was inserted through the nostril for a depth of 1-2 mm. The pump was actuated and the powder was emitted in one shot. Immediately after use, the device was re-weighed to determine the quantity of powder emitted and calculate the actual dose administered.
The time points of interest for measuring flurbiprofen levels in the brain were set at 5, 10, 20 and 30 min after treatment. For the purpose, the rats in each treatment group were

divided into the corresponding four subgroups, one per time point (number of animals per subgroup ≥3) The brain was collected after cervical dislocation and total body perfusion with cold PBS pH 7.4 (5 min, 120 ml) to remove residual blood.

Blood samples were also taken via puncture of the lateral vesicular vein at all specified time points until the animal sacrifice. Blood samples were collected in non-heparinized Eppendorf tubes and immediately centrifuged to separate serum. Serum and brain samples were frozen and stored at -70 °C until extraction and HPLC analysis.

2.5 Flurbiprofen Extraction from Biological Samples

The procedure to extract flurbiprofen from the biological samples was adapted from Christodoulou et al. (2015), using ibuprofen as internal standard.

2.5.1 Flurbiprofen extraction from rat serum

0.5 ml of ibuprofen solution in acetonitrile (0.7 mg/ml) and 0.05 ml of methanol were added to 0.25 ml of serum sample and vortexed for 15 sec. After centrifugation (10 min, 7500 rpm, 20 °C) to precipitate the plasma proteins, the clear supernatant was analyzed to quantify flurbiprofen as such or after dilution with blank serum, when flurbiprofen concentration in serum exceeded the linearity range of the analytical method. The drug extraction efficiency from serum samples was assessed in samples containing flurbiprofen concentrations ranging from 5 to 1260 ng/ml. Drug recovery was 100% in the range 90-1260 ng/ml flurbiprofen concentration in serum samples.

2.5.2 Flurbiprofen extraction from rat brain

After the animal's death, the rat's body was perfused with 120 ml of cold PBS pH 7.4 to remove the blood from the vessels. To do so, the abdominal area was disinfected with ethanol 70% (v/v), then opened with a surgical blade. The caudal vena cava was catheterized and 5 ml of blood were immediately withdrawn with a 10 ml syringe. Then, the xiphoid cartilage was lifted up, the chest opened, and the pleura removed to release the heart. PBS was perfused at 24 ml/min rate by means of a Watson Marlow 323 peristaltic pump (IT-Mazzano, BS) connected with a 23G butterfly needle inserted into the heart's left ventricle. After perfusion, the brain was dissected from the head, rinsed with water for injection, weighed and frozen (-70 °C) into a plastic container. For the IV group, the whole brain was frozen without isolating the olfactory bulb. Conversely, for the rats receiving

flurbiprofen intranasally (IN groups), the olfactory bulb was isolated for quantifying the drug independently of the rest of the brain.

On the day of analysis, the brain (or bulb) was thawed at room temperature and homogenized with a T10 ULTRA-TURRAX® (IKA Werke, DE-Staufen im Breisgau) in presence of a measured volume of PBS pH 7.4 (tissue:PBS ratio 1:2 w/w). For the isolated bulb, homogenization in PBS pH 7.4 was carried out in a 2-ml Eppendorf® microtube by smashing the tissue with a disposable polypropylene pestle (Sigma-Aldrich, St. Louis, MO, USA). The resulting tissue homogenate was centrifuged to remove the coarse material (3 min, 3000 x g, 20 °C). Flurbiprofen was extracted from the supernatant following the same procedure adopted for serum, then quantified by HPLC analysis.

2.6 HPLC-FLD Method for Flurbiprofen Quantification in Biological Samples

Flurbiprofen in biological samples was quantified by reverse-phase HPLC with fluorescence detection (HPLC-FLD; Shimadzu, JP-Kyoto). Isocratic elution was carried out with a NaH_2PO_4 20 mM:CH_3CN (40:60) mobile phase (pH 3.0 $\pm$ 0.1) at 30 °C. The detection wavelength was set at 254 nm and 308 nm for excitation and emission, respectively. The column was a ZORBAX Eclipse XDB (C18, 5 µm, 4.6 x 150 mm; Agilent, Santa Clara, CA, USA). The flow rate was 1 ml/min and injection volume 20 µl. In these conditions, the retention time of flurbiprofen was 3.9 min, while the internal standard (ibuprofen) was eluted at 5.1 min. The method was developed in-house and validated with respect to linearity, repeatability, matrix effect, limit of quantification (LOQ) and limit of detection (LOD).

Stock solutions of flurbiprofen (0.5 mg/ml) and internal standard (ibuprofen, 0.7 mg/ml) were prepared in acetonitrile and stored at 2-8 °C for up to 2 weeks before use. Standard solutions of flurbiprofen in the range 3-1300 ng/ml, with the internal standard at 24 µg/ml fixed concentration, were prepared by dilution of aliquots of flurbiprofen and ibuprofen stock solutions with acetonitrile and used for the construction of the calibration curves in rat serum and brain homogenate in PBS pH 7.4.

Linearity was confirmed in the considered flurbiprofen concentration range both for serum and tissue samples. The effect of the biological matrix on the slope and intercept of the calibration curve was not influential comparing the curves in serum with those in brain homogenate. Method repeatability was assessed by six consecutive injections of samples at 3 ng/ml, 95 ng/ml, 1260 ng/ml flurbiprofen and 24 µg/ml ibuprofen in serum. The Relative standard deviation (RSD) resulted equal to 3.2, 0.4 and 0.31 for the lowest,

intermediate and highest flurbiprofen concentration, respectively. The repeatability of calibration curves was also assessed, both inter- and intra-day. The calibration curves were always superimposable. Limit of quantification (LOQ) and limit of determination (LOD) were calculated based on the “Standard Deviation of the Response and the Slope” approach (European Medicines Agency, 1995). LOQ was 5.80 ng/ml and 3.87 ng/ml, while LOD was 1.91 ng/ml and 1.28 ng/ml, respectively in rat serum and brain homogenate in PBS pH 7.4.

2.7 Non-Compartmental PK Analysis

Sparse sampling non-compartmental PK analysis (NCA) was performed for all *in vivo* data using Phoenix® 7.0 (Certara, Princeton, NJ, USA), to determine serum and brain PK parameters, namely area under the curve ($AUC_{0\text{-}t}$), maximum concentration or peak (C_{max}) and time-to-peak (t_{max}), and to calculate the absolute bioavailability of flurbiprofen after intranasal (IN) administration of powders and solution. The NCA sparse method calculates PK parameters based on the mean profile for all the subjects in the data set. In addition, it uses the subject information to calculate standard errors that will account for any correlations in the data resulting from repeated sampling of individual animals. The linear-log trapezoidal method was used to calculate $AUC_{0\text{-}t}$. The absolute bioavailability of flurbiprofen after IN administration was calculated by comparing AUCs after IN and intravenous (IV) administration according to Equation 1:

$$\frac{AUC_{0-t\ (IN)} \times Dose_{(IV)}}{AUC_{0-t\ (IV)} \times Dose_{(IN)}} \qquad \text{Eq. 1}$$

where $AUC_{0\text{-}t\ (IN)}$ and $AUC_{0\text{-}t\ (IV)}$ are the area under the concentration *vs.* time curve from 0 to the last sampling time after IN and IV administration, respectively. $Dose_{(IN)}$ and $Dose_{(IV)}$ are the respective administered doses.

Similarly, Equation 1 was applied to analyze the flurbiprofen concentrations measured in the brain (brain disposition).

2.8 Statistical Analysis

Data are expressed mean ± SEM (standard error of the mean). They were compared by applying an unpaired two-tailed Student’s t-test. $p<0.05$ was considered to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1 Flurbiprofen Sodium Nasal Powders

3.1.1 Spray-dried microparticles and soft pellets

Flurbiprofen sodium spray-dried microparticles for nasal insufflation have been described in a previous paper (Tiozzo Fasiolo et al., 2019). A dry powder product for nasal deposition is a combination of the drug formulation with a nasal insufflator. Both components contribute to the efficiency of the delivery during insufflation. In this work, the nasal formulation of flurbiprofen sodium spray-dried microparticles has been studied in comparison with the same microparticles agglomerated in soft pellets. Soft pellets have proved to be suitable for combination with insufflator devices for nasal powder (Balducci et al., 2013; Giuliani et al., 2018; Russo et al., 2006), since their free-flowing characteristics facilitate the dose metering and emission. During insufflation into the nose, the air flow turbulence applied by the device breaks the soft pellets into fragments (Giuliani et al., 2018). These fragments, composed of several microparticles, have suitable size for nasal deposition by impaction on the epithelium; at the same time, they mitigate the risk of lung entrance (Russo et al., 2004). After deposition, in contact with the nose mucosal fluid, the soft pellet fragments disaggregate restoring the primary microparticles that quickly dissolve (Balducci et al., 2013; Raffin et al., 2007; Russo et al., 2004; Russo et al., 2006).
Among the microparticulate powders described in the previous paper (Tiozzo Fasiolo et al., 2019), two powders of flurbiprofen sodium spray-dried at different temperature with the Nano spray dryer B-90 (coded F13_70_MP and F13_40_MP), were selected for the *in vivo* animal study (Fig. S1 in Supplementary Material). Unfortunately, these flurbiprofen powders did not spontaneously agglomerate. As shown by Giuliani et al. (2018), mannitol microparticles containing lecithin as binding agent, here made by means of the Nano B-90-spray dryer, easily agglomerated. Therefore, 1:1 blends of flurbiprofen sodium (FB-COONa) spray-dried microparticles and mannitol/lecithin spray-dried microparticles were prepared. By tumbling these mixtures, soft pellets containing flurbiprofen sodium were constructed in size range 106-500 µm.

Table I. Soft pellets of flurbiprofen sodium (FB-COONa) and excipient spray-dried microparticles mixtures (size range 106-500 µm).

FB-COONa microparticles	FB-COONa/excipient ratio	FB-COOH content (% w/w)
F13_70_MP	50:50	34.6 ± 2.3
F13_40_MP	50:50	35.0 ± 0.7

Table I shows the composition of the two soft pellet powders prepared from FB-COONa Nano B-90 spray-dried microparticles blended with mannitol/lecithin microparticles. The two agglomerated powders were similar in terms of manufacturing yield (≥75%) and drug content. However, only the F13_40 soft pellets (F13_40_SP) were used for the *in vivo* tests because of the higher homogeneity of drug content (Fig. 1).

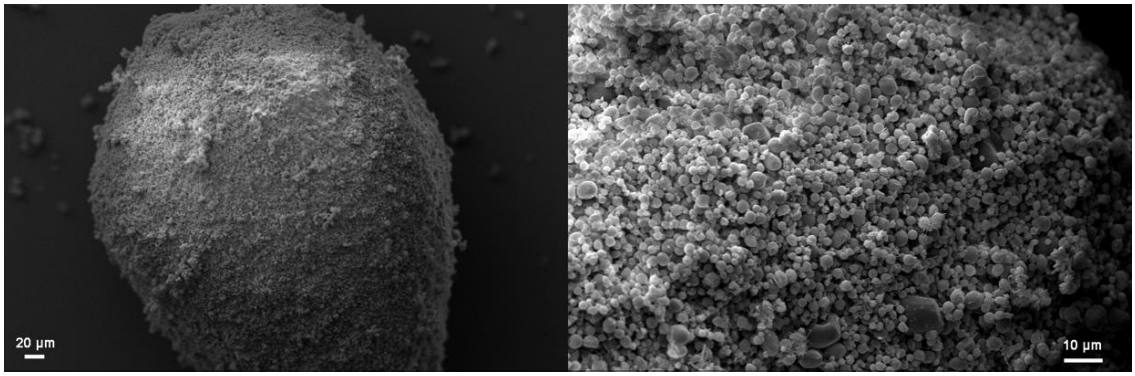


Figure 1. SEM micrographs of (from left to right): F13_40_SP soft pellet (500x) and a detail of its surface (2000x).

3.1.2. Soft pellet *in vitro* dissolution and *ex vivo* permeation across nasal mucosa

The *in vitro* dissolution rate of flurbiprofen sodium soft pellets, made from the mixture with excipient microparticles, was measured in Franz-type diffusion cells using a wet regenerated cellulose membrane as barrier between donor and receptor compartments. Despite the flurbiprofen content in the pellets was diluted by the excipient microparticles, the dissolution rate of the soft pellets was slightly higher than the corresponding primary microparticles (Fig. S2 in Supplementary Material). The fraction of flurbiprofen dissolved within the first 30 min was between 20-40%. Thus, the presence of excipient microparticles in the soft pellets' composition positively impacted on flurbiprofen sodium dissolution in the selected experimental set-up.

Successively, the *ex vivo* drug permeation across rabbit nasal mucosa was tested for soft pellets manufactured with F13_70 or F13_40 flurbiprofen sodium microparticles and compared with the corresponding drug microparticle powders alone. Powder amounts equivalent to about 4 mg of FB-COOH were manually deposited on the nasal mucosa barrier at the bottom of the Franz cell donor, paying attention to uniformly distribute the sample. In one hour, considered a reasonable time limit for embracing the powder permanence inside the nose of an insufflated formulation, the amount of drug permeated

from all formulations was between 11-26% of the loaded amount. Figure 2 shows that the flurbiprofen permeation profiles from soft pellets, made with the two microparticle formulations, had similar rate; however, the corresponding primary drug microparticles (non-agglomerated) in the first hour led to significantly different permeation: the F13_70 soft pellets had a significantly higher permeation profile than the corresponding primary drug microparticles ($p<0.05$). In contrast, for the F13_40 soft pellets, the flurbiprofen amount permeated in the first hour was lower than from the corresponding microparticles, but the difference was not statistically significant ($p=0.38$).

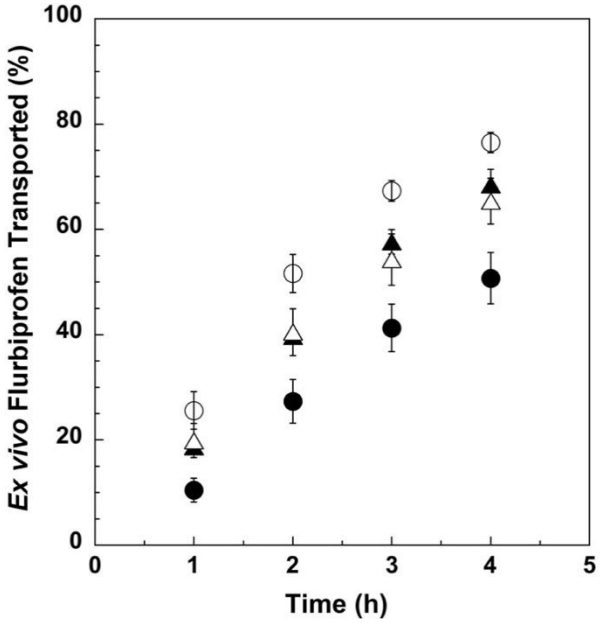


Figure 2. Flurbiprofen transport across rabbit nasal mucosa from soft pellets (triangle) of F13_40_MP (white) and F13_70_MP (black) vs. the primary microparticles (circle) F13_40_MP (white) and F13_70_MP (black) (mean ± SEM, n≥5). Data for the microparticles have been re-elaborated from Tiozzo Fasiolo et al. (2019).

In our previous research, the different *ex vivo* permeation of primary microparticles spray-dried at 70 °C and 40 °C was attributed to the differences in their particle size and crystallinity (Tiozzo Fasiolo et al., 2019). Blending and agglomeration with the excipient microparticles eliminated such permeation differences between the two drug microparticle formulations. However, the soft pellets behaved differently in terms of flurbiprofen permeation across the mucosa as compared to the corresponding microparticles alone. After having seen the performance of soft pellets compared to microparticles in the dissolution tests (Fig. S2 in Supplementary Material), an additional influential action on flurbiprofen permeation rate from the soft pellet formulation in contact with the slightly wet mucosa was envisaged.

Nevertheless, even taking into account the difference in size and composition between primary microparticles and soft pellets, the latter did not exhibit substantially different drug permeation, in particular in the first hour.

3.2 Powder Combination with the Insufflator Device

For the construction of the nasal product, the F13_70 flurbiprofen sodium spray-dried microparticles and the soft pellets made of F13_40 flurbiprofen sodium microparticles were combined with a nasal insufflator. The Aptar's Unidose Powder System (UDS), an active device, was selected to deliver the powders by insufflation. The UDS is used in a prescription drug approved in 2019 by the U.S. FDA for an intranasal rescue treatment for severe hypoglycemia in diabetic people (Aranishi et al., 2020; Suico et al., 2020). This device is specifically designed for drug deposition in the upper part of the human nasal cavity (olfactory region), favoring drug nose-to-brain transport. In addition, the device could be adapted to rat nose anatomy because a special tip to fit the device to rat nose was provided.

The device performance was assessed by measuring the emitted amount of powder following its activation in one nostril of the rat's nose during the PK study. Considering the powder masses loaded (about 13 mg of spray-dried microparticles or 15 mg of soft pellets), the insufflator emitted 65% or 83% of the loaded powder, respectively (Table II). It was evident that the efficiency of powder delivery into the nose by the UDS device was superior when loaded with soft pellets as compared to microparticles. The soft pellets coarse size facilitated not only the powder dosing in the insufflator reservoir, but also its delivery.

In the nasal product preparation for the study in rats, the amount of drug powder to insufflate was limited by the dimension of rat nose. Thus, the dose of FB-COOH for brain uptake via nasal route in rat was 4.2 and 6.7 mg, respectively with the soft pellets and the microparticles.

Table II. Nasal insufflation in rats from UDS powder device of flurbiprofen sodium spray-dried microparticles (F13_70_MP) and soft pellets of flurbiprofen sodium spray-dried microparticles with excipient microparticles (F13_40_SP). Data are reported as mean ± standard deviation (n≥13).

Nasal Powder	Powder Loaded (mg)	Powder Emitted (mg)	FB-COOH Emitted (mg)
F13_70_MP (Microparticles)	12.9 ± 0.9 (10.4 mg FB-COOH)	8.4 ± 1.2 (65%)	6.7 ± 1.0
F13_40_SP (Soft pellets)	14.6 ± 0.7 (5.1 mg FB-COOH)	11.9 ± 1.1 (83%)	4.2 ± 0.4

Since these values of powder delivery were collected during the actual administration to rats, we assumed that the amounts of flurbiprofen emitted, and reported in Table II as flurbiprofen acid (active moiety), represented the amount of drug deposited into nose.

3.3 Pharmacokinetics in Rat After Nasal Administration

Drug absorption into blood across the nasal epithelium occurs by transcellular or paracellular pathways in both the respiratory and olfactory nasal regions (Dhuria et al., 2010). Flurbiprofen is a low molecular weight drug (MW 244.2 g/mol), supporting the transport through the nasal mucosa by both pathways (Lochhead and Thorne, 2012). Following the olfactive or trigeminal nerve routes, a direct transport of drug to brain could also take place along these nervous structures.

To study flurbiprofen absorption from the nose and disposition in the brain, the two nasal powders previously selected, namely F13_70 microparticles and soft pellets of F13_40 microparticles, were insufflated into the nose of rats. The amount of powder loaded in the nasal device and the corresponding flurbiprofen dose emitted and insufflated into the rat nose are reported in Table II. The powder amount manually metered in the insufflator reservoir ranged between 12-15 mg, complying with the objective to administer similar masses of powder. However, the presence of the excipients used for agglomeration, reduced the dose of flurbiprofen administered with soft pellets, as compared to the same mass of microparticle powder. Consequently, also due to the different amount of powder emitted, the doses of flurbiprofen deposited resulted different. By weighing the insufflator (sensitivity 0.01 mg) before and after the administration, the amount of flurbiprofen made available by the insufflation of microparticles or soft pellets in one nostril was calculated as 6.7 mg and 4.2 mg, respectively (see Table II). Finally, for determining the fraction absorbed and brain disposition, intravenous and intranasal solutions of flurbiprofen sodium were administered as well.

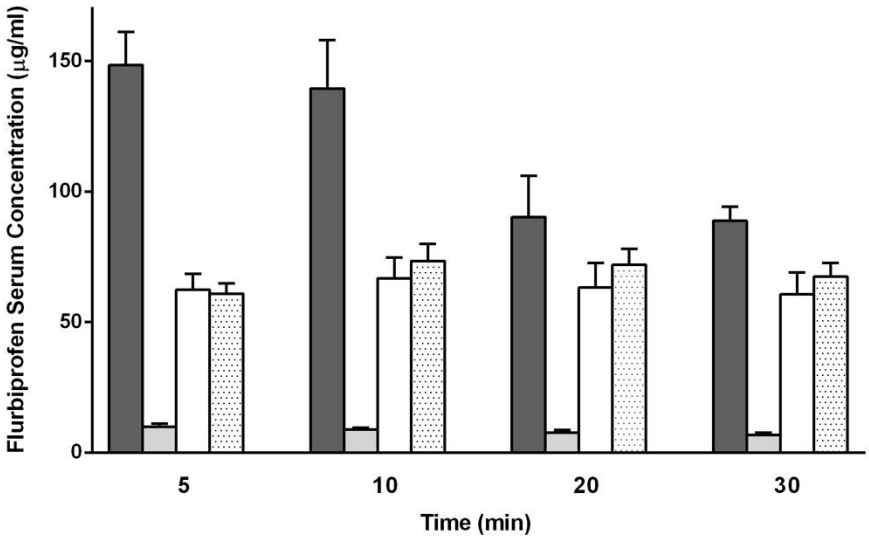


Figure 3. Flurbiprofen serum concentration vs. time after intravenous (IV; dark grey) and intranasal (IN) administrations: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white). Data are expressed as mean ± SEM (n≥3).

The flurbiprofen serum profiles obtained with the four dosage forms administered, are illustrated in Figure 3. The device loaded with flurbiprofen spray-dried microparticles contained a metered dose higher than the soft pellet-loaded device; thus, despite the lower emitted fraction, a higher dose of FB-COOH was insufflated (Table II). The serum levels in Figure 3 are not dose-normalized based on the amount of flurbiprofen intranasally emitted. The insufflation of the nasal powders gave rise to a very rapid flurbiprofen nasal absorption, with important presence in blood already at 5 min; C_{max} was achieved within 10 min after insufflation of microparticle or soft pellet powders (C_{max} 66.8 ± 7.9 μg/ml and 73.4 ± 6.5 μg/ml, respectively). The C_{max} of flurbiprofen serum profiles of the two powders were quite close. In fact, despite the 37% difference in the nominal dose, the drug concentrations of microparticles and soft pellets in serum (Fig. 3) were not significantly different ($p>0.05$). Eventually, thirty minutes after insufflation, flurbiprofen serum concentrations of microparticles or soft pellets decreased to 60.6 ± 8.4 μg/ml and 67.4 ± 5.2 μg/ml, respectively. In summary, the microparticle agglomerated in soft pellets improved the metering and emission of the nasal powder, without significantly affecting the rapid drug release and absorption from the microparticles.

In contrast, faster flurbiprofen systemic absorption was observed after nasal administration of the flurbiprofen solution, as compared to nasal powders, with C_{max} reached within 5 min. This was rather expected because, being the drug already dissolved, the systemic absorption was not limited by the powder dissolution process. Moreover, in this experiment

the nasal surface for drug absorption was doubled, because two nostrils were engaged during the solution application. However, being limited by flurbiprofen sodium aqueous solubility, the administered flurbiprofen dose was 10 to 20 times as lower as compared to nasal powders. Moreover, the small airway volume of the rat nasal cavity (0.2 cm^3; (Xi et al., 2016)) allowed for the administration of maximum 10 µl of drug solution per rat nostril. Accordingly, after flurbiprofen nasal solution administration (0.3 mg drug dose), the obtained serum C_{max} was 10.0 ± 1.1 µg/ml (Fig. 3), that was approximately seven-fold lower than the values measured after nasal powder administration.

The AUC_{0-t}, i.e., the body exposure to flurbiprofen, despite the lower dose administered, exhibited a superior value for the soft pellets as compared to microparticles, but the difference was not statistically significant (p=0.45) (Table III).

In contrast, the FB-COOH dose of intranasal solution was largely lower than nasal powder dose. Consequently, the relative AUC_{0-t} was the lowest, quite in line with the powder to solution dose ratio.

In summary, taking as reference the intravenous administration of 4.5 mg of FB-COOH, the nasal insufflation of 6.7 mg of drug as microparticles and of 4.2 mg as soft pellets gave rise to notable fractions of drug absorbed in blood, reaching for the soft pellets the highest absolute bioavailability between the nasal powders, i.e., 58.1% versus 33.3%, respectively. Nevertheless, the intranasal solution exhibited the highest fraction absorbed value (96.2%). In this last case, the dose was accurately administered by dropping the solution with a pipette in two rat nostrils, whereas only one nostril was engaged with powder insufflation. The higher bioavailability value of soft pellets compared to microparticles, signaled that, despite the lower dose, the deposition of soft pellets for systemic absorption was more effective. Moreover, an influence on drug absorption by the excipients used for agglomeration, as seen also in the *ex vivo* permeation studies, cannot be excluded. In this regard, it has been shown that mannitol alone and in combination with temozolomide, increased the permeability of two different-sized fluorescent tracers across a blood brain barrier cell model (Choi et al., 2018). This was attributed to a decreased expression of tight junction proteins. Tight junction proteins are present also in the nasal epithelium.

3.4 Flurbiprofen Brain Disposition

The flurbiprofen brain disposition can be the result of drug transport across the BBB and direct brain passage through the nerves and perineural space of olfactory and respiratory

epithelia (Inoue et al., 2020; Lochhead and Thorne, 2012). In this study, both the systemic and local administration routes enabled an amount of flurbiprofen to access to the central nervous system (Fig. 4A). After injection of 4.5 mg of flurbiprofen, a maximum level of 0.63 ± 0.03 μg/g brain tissue was reached within ten minutes. Interestingly, the highest brain concentration was measured after intranasal administration of 6.7 mg of FB-COOH as microparticulate powder (C_{max} 2.0 ± 0.8 μg/g tissue). Among all administrations, the superior brain levels obtained with the nasal microparticles were maintained at all time points. Differently from serum levels, that were quite similar for the two powders, the soft pellets (4.2 mg of FB-COOH) gave lower brain concentrations than the microparticle powder (C_{max} 1.3 ± 0.5 μg/g tissue). Due to the high inter-animal variability, the differences were not statistically significant ($p>0.05$).

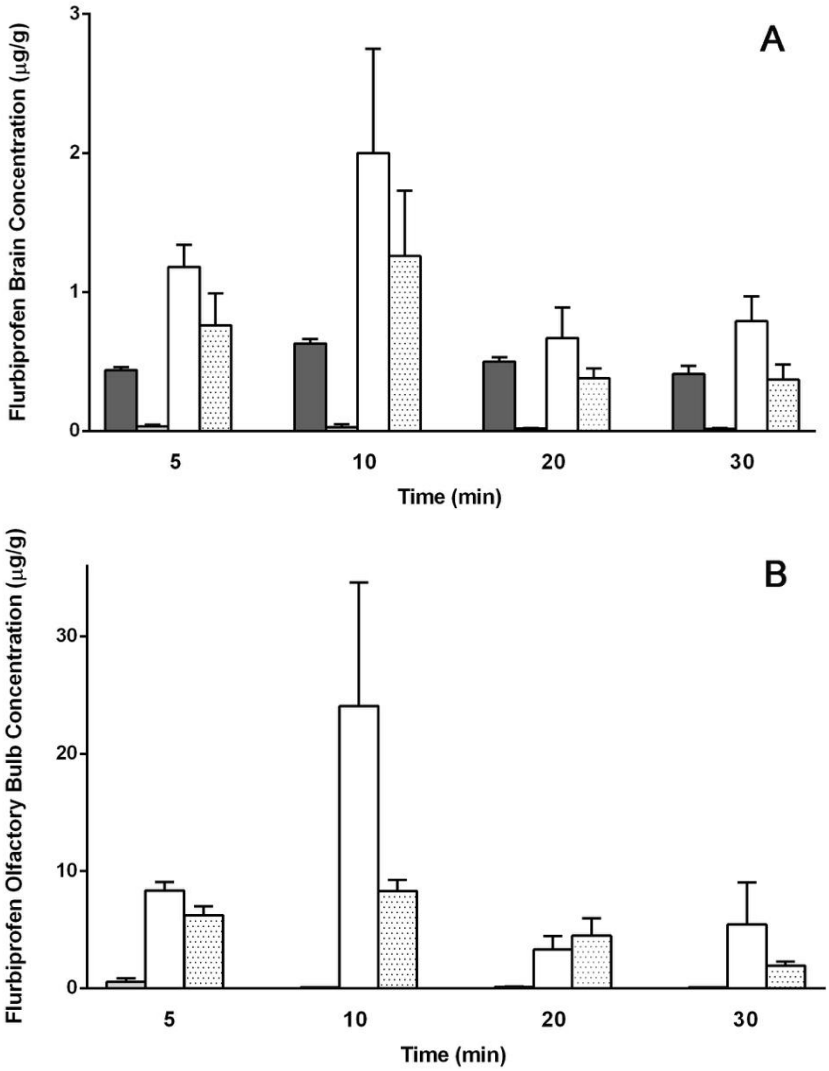


Figure 4. Flurbiprofen concentration in brain (panel A) and olfactory bulb (panel B) after intranasal (IN) administration of: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white)

vs. intravenous injection (IV; dark grey). Flurbiprofen levels in olfactory bulb were not measured for the IV treatment. Data are expressed as mean ± SEM (n≥3).

By evaluating serum and brain levels together, it is reasonable to deduce that the differences in flurbiprofen brain levels after systemic (IV) and local (IN) administration of nasal powders imply a direct nose-to-brain transport. In fact, the higher serum levels following IV injection (compared to serum levels with both nasal powders), did not reflect into higher brain levels.

Moreover, comparing the two nasal powders, the brain disposition was somehow different, in spite of the substantially similar serum levels obtained. Although the brain levels were never significantly different, the superiority of the microparticles suggests an effect of powder particle size on flurbiprofen nose-to-brain transport, since the contribution of BBB passage to drug brain availability should be the same at similar serum levels.

The brain disposition of FB-COOH administered as nasal solution was very low, but the dose was also 15-20 times as lower.

As a result of intranasal insufflation of flurbiprofen sodium powders, very significant drug concentrations were measured in the olfactory bulb as compared to rest of the brain (Fig. 4B). As this compartment is directly connected to the nasal cavities, drug presence in the bulb signifies direct nose-to-brain transport after intranasal administration.

The findings confirm that flurbiprofen administered intranasally as powder form, can directly reach the brain through the nose-to-brain pathway. Moreover, the very high concentration in the olfactory bulb represents a promising aspect of flurbiprofen brain targeting in Alzheimer's disease. In fact, olfactory impairment is recognized as an early sign of AD and other neurodegenerative disorders. It is caused by morphological and signaling alterations of the olfactory nerve (Brai and Alberi, 2018) and correlates with cognitive impairment development. According to Bathini et al. (2019), this suggests that neuronal network imbalances propagate via olfactory bulb and nerve to higher brain centers of the entorhinal cortex and hippocampus. AD initiates in the entorhinal cortex and then spreads outward in an anatomically defined pattern (Adams et al., 2019; Bathini et al., 2019; Holbrook et al., 2020).

In summary, the nasal administration of powders enables a significant presence of flurbiprofen in the central nervous system where it is expected to be therapeutic in AD. With these nasal powder dosage forms, it is possible to attain a concentration of flurbiprofen in the brain superior to the one obtained *via* systemic delivery (Lehrer, 2014).

After intranasal powder administration, the flurbiprofen accumulation in brain was envisaged to come from the dual contribution to entry through the blood brain barrier (as for IV administration) and through the nose-to-brain direct pathway. Examining the brain disposition, also reported in Table III, flurbiprofen nasal microparticulate powder, in addition to a fraction arrived through the BBB, made available a significant amount of drug directly through the olfactory area in the nasal cavity.

Finally, with the nasal solution, FB-COOH presence in the brain at all time points was about 50-fold lower than with the nasal powders. The maximum concentration in the olfactory bulb was 0.55 ± 0.29 µg/g tissue.

2.8 Data Analysis by Nose-to-brain Delivery Indexes

The direct brain transport contribution after intranasal administration can be evaluated by dedicated PK parameters used to quantify the efficiency of nose-to-brain direct delivery. For this evaluation, Drug Targeting Efficiency Percentage (DTE) and Nose-to-Brain Direct Transport Percentage (DTP) indexes have been reviewed by Kozlovskaya et al. (2014). Assuming a linear PK of flurbiprofen (Szpunar et al., 1987), Drug Targeting Efficiency Percentage expresses the brain drug exposure relative to blood exposure after intranasal administration, compared to the brain exposure relative to blood drug exposure after intravenous administration, according to Equation 2:

$$DTE = \frac{\left(\frac{AUC_{0-t(brain)}}{AUC_{0-t(blood)}}\right)_{IN}}{\left(\frac{AUC_{0-t(brain)}}{AUC_{0-t(blood)}}\right)_{IV}} * 100 \qquad \text{Eq. 2}$$

where $AUC_{0\text{-}t(brain)}$ and $AUC_{0\text{-}t(blood)}$ are the area under the concentration *vs.* time curve of flurbiprofen in brain and in blood, respectively, following intranasal (IN) and intravenous (IV) administrations. DTE values range between 0 and infinitive; values higher than 100 indicate a brain drug uptake more efficient by IN than by IV administration.

Additionally, the Direct Transport Percentage index estimates the fraction of intranasal dose reaching the brain via direct nose-to-brain transport *vs.* the total amount of drug found in the brain following the intranasal delivery, according to Equation 3:

$$DTP = \frac{B_{IN} - B_x}{B_{IN}} * 100 \qquad \text{Eq. 3}$$

where B_{IN} is the $AUC_{0\text{-}t(brain)}$ following intranasal administration and B_x is the portion of the same $AUC_{0\text{-}t(brain)}$ accounting for the drug amount that entered the brain via systemic circulation (i.e., crossing the BBB). Bx can be calculated according to Equation 4:

$$B_x = \frac{B_{IV}}{P_{IV}} . P_{IN} \qquad \text{Eq. 4}$$

where B_{IV} is the brain $AUC_{0\text{-}t(brain)}$ and P_{IV} the $AUC_{0\text{-}t(blood)}$ of intravenous administration; P_{IN} is the $AUC_{0\text{-}t(blood)}$ of intranasal administration.

According to Kozlovskaya et al. (2014), the value of DTP can range from -∞ to 100. However, we believe that values equal to zero or negative indicate that the drug is delivered to the brain essentially *via* BBB.

The interest of these values is that they are independent of the different doses administered. In this study, DTE values of the nasal powders were notably higher than 100, identifying a more efficient nasal brain targeting compared to IV injection. In contrast, the nasal solution was less efficient, exhibiting a value lower than 100 (Table III). In detail, following intranasal powder administration, the brain targeting efficiency was more consistent with the nasal microparticles than with the soft pellets, i.e., DTE 456% vs 251%, respectively.

The Direct Transport Percentage index measures the fraction of intranasal dose entered the brain directly via nose-to-brain passage out of the total amount reaching the brain *via* any route including BBB crossing. The negative value of DTP for the intranasal solution indicates there was no direct nose-to-brain transport. Conversely, the DTP values for both nasal powders were higher than 60%. Thus, the intranasal powder administration added to the BBB contribution a relevant direct flurbiprofen transport from the olfactory region to the brain. In summary, the flurbiprofen sodium nasal powders revealed to be suitable formulations for an efficient direct transport to brain following their nasal insufflation.

Table III. Area Under the Curve (AUC) calculated in serum and in brain, Drug Targeting Efficiency (DTE) and Direct Transport Percentage (DTP) of flurbiprofen for intranasal drug powders, namely F13_70 microparticles and F13_40 soft pellets, and drug solution compared to IV administration. AUC data are expressed as mean ± SEM.

Treatment	**$AUC_{0\text{-}t}$ serum** ($\mu g\ ml^{-1}\ min$)	**$AUC_{0\text{-}t}$ brain** ($\mu g\ g^{-1}\ min$)	**DTE** (%)	**DTP** (%)
IV solution	3528.5 ± 184.8	13.97 ± 0.50	-	-

F13_70_MP	1748.4 ± 171.5	31.56 ± 6.14	456	78
F13_40_SP	1912.0 ± 113.2	18.99 ± 3.79	251	60
IN solution	226.3 ± 22.2	0.65 ± 0.18	72	-39

The microparticulate powder enabled a higher direct traffic of flurbiprofen from the nasal cavity to the brain than soft pellets. The nose-to-brain direct transport relies on deposition and retention of the powder at olfactory region. With soft pellets, in which the drug is diluted by excipients, the drug amount deposited per unit epithelium area is expected to be less favorable. The high bioavailability obtained with the soft pellets powder suggests an important deposition in the respiratory region. Better coverage of the olfactory mucosa by the microparticulate pure drug powder may have favored nose-to-brain transport. It is known that the shape of the plume emitted from a device and the deposition of particles within nasal cavities are influenced by the properties of powder formulation (Buttini et al., 2012). This leads to different particle lining of the mucosal surface, either respiratory or olfactory. Soft pellets have lower aerosolization performance in terms of de-agglomerated particle size emitted by nasal device, compared to microparticles. In fact, in a study regarding the technological development of soft pellets of caffeine spray-dried microparticles for nasal delivery, Russo and co-workers (2004) reported that during insufflation the agglomerates were broken in fragments with significantly reduced size. Still these fragments were larger than the original microparticles. More specifically, the size of fragments was dependent on the agglomerate's mechanical resistance (Adi et al., 2011). This size difference ultimately affects the site of drug dissolution and transepithelial transport (Buttini et al., 2012; Tiozzo Fasiolo et al., 2018).

Concerning the liquid dosage form, the nasal solution was less efficient than the nasal powders in direct delivery of flurbiprofen to the brain, having DTE <100 and negative DTP, the latter indicating a negligible nose-to-brain direct uptake. However, the amount of flurbiprofen solution was inappropriate to persistently cover the rat nasal olfactory area; dose application by dropping has likely resulted in deposition primarily in the respiratory epithelium (anterior part of nasal cavity) with poor involvement of the olfactory epithelium. In agreement with Tanaka et al. (2016), the nasal solution seemed less effective than the powders at enabling drug access to the brain. In our study, the high bioavailability and the unfavorable physical form to maintain the drug in contact with the olfactory epithelium, are evoked in interpreting the different liquid/powder behavior.

CONCLUSIONS

The nasal insufflation of flurbiprofen sodium powders, both in form of microparticles or soft pellets constructed with excipient microparticles, in addition to BBB transport, revealed a direct drug transport to brain from the olfactory region.

Compared to intravenous administration, flurbiprofen sodium powders, insufflated into the nose, enhanced the drug concentration in brain, despite the lower drug serum concentration. The Direct Transport Percentage index evidenced that at least 60% of the intranasal dose reached the brain via direct nose-to-brain transport for both powders. Nasal soft pellets, very effective in dose delivery, showed a fraction of drug absorbed through the respiratory epithelium, higher than the primary microparticles. However, nasal microparticle powder outperformed the soft pellet powder in the direct transport of flurbiprofen to brain. The very high drug concentration in the olfactory bulb measured for microparticulate powders, substantiates the direct nose-to-brain drug transport. The deposition of microparticles by nasal insufflation into rat nasal cavity resulted in larger surface of olfactory mucosa covered by impacted particles, hence, sustaining the drug passage to brain along olfactory epithelium.

The drug solution was not effective in direct nose-to-brain transport compared to microparticles based solid dosage forms. The small amount of drug intranasally instilled as solution was mainly absorbed to blood, indicating a marginal retention on olfactory epithelium.

Also considering the difference between microparticles and soft pellets in brain direct access, the impaction and deposition of drug particles on olfactory mucosa has to be the relevant mechanism for the nose-to-brain transport by administering nasal powders. In addition, the powder dissolution on site provides a high and long-lasting concentration gradient. The relevant concentrations of flurbiprofen in brain olfactory bulb, due to the bulb connection with the entorhinal cortex from where Alzheimer's disease initiates (Holbrook et al., 2020), pushes further investigations in an Alzheimer's disease animal model of the flurbiprofen sodium nasal powders.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Aptar Pharma for donating the Unidose Powder System device for the *in vivo* experiments.

This paper is dedicated to the memory of prof. Angelo Scatturin, a warm-hearted, well-respected professor of Pharmaceutical Technology, who had two loves in life: his family and the university. His students and collaborators remember him with heartfelt gratitude.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

COMPETING INTEREST STATEMENT

The authors declare no competing interests.

REFERENCES

Adams, J.N., Maass, A., Harrison, T.M., Baker, S.L., Jagust, W.J., 2019. Cortical tau deposition follows patterns of entorhinal functional connectivity in aging. eLife. 8, e49132. https://doi.org/10.7554/eLife.49132

Adi, S., Adi, H., Chan, H.K., Finlay, W.H., Tong, Z., Yang, R., Yu, A., 2011. Agglomerate strength and dispersion of pharmaceutical powders. J. Aerosol Sci. 42, 285-294. https://doi.org/10.1016/j.jaerosci.2011.02.002

Ali, M.M., Ghouri, R.G., Ans, A.H., Akbar, A., Toheed A., 2019. Recommendations for Anti-inflammatory Treatments in Alzheimer's Disease: A Comprehensive Review of the Literature. Cureus. 11, e4620. https://doi.org/10.7759/cureus.4620

Ambrus, R., Gieszinger, P., Gáspár, R., Sztojkov-Ivanov, A., Ducza, E., Márki, A., Janáky, T., Tömösi, F., Kecskeméti, G., Szabó-Révész, P., Bartos, C., 2020. Investigation of the Absorption of Nanosized lamotrigine Containing Nasal Powder via the Nasal Cavity. Molecules. 25, 1065. https://doi.org/10.3390/molecules25051065

Aranishi, T., Nagai, Y., Takita, Y., Zhang, S., Nishimura, R., 2020. Usability of Nasal Glucagon Device: Partially Randomized Caregiver and Third-Party User Experience Trial with Simulated Administration at a Japanese Site. Diabetes Ther. 11, 197–211. https://doi.org/10.1007/s13300-019-00711-1

Balducci, A.G., Ferraro, L., Bortolotti, F., Nastruzzi, C., Colombo, P., Sonvico, F., Russo, P., Colombo, G., 2013. Antidiuretic effect of desmopressin chimera agglomerates by nasal administration in rats. Int. J. Pharm. 440, 154-160. https://doi.org/10.1016/j.ijpharm.2012.09.049

Bathini, P., Mottas, A., Jaquet, M., Brai, E., Alberi, L., 2019. Progressive signaling changes in the olfactory nerve of patients with Alzheimer's disease. Neurobiol. Aging. 76, 80-95. https://doi.org/10.1016/j.neurobiolaging.2018.12.006

Brai, E., Alberi, L., 2018. Olfaction, among the first senses to develop and decline, in: T. Heinbockel (Ed.), Sensory Nervous System, IntechOpen Ltd, London, pp. 65. https://doi.org/10.5772/intechopen.75061

Buttini, F., Colombo, P., Rossi, A., Sonvico, F., Colombo, G., 2012. Particles and powders: tools of innovation for non-invasive drug administration. J. Control. Release. 161, 693-702. https://doi.org/10.1016/j.jconrel.2012.02.028

Choi, C., Kim, H.M., Shon, J., Park, J., Kim, H., Oh, S., Kim, N.K., Kim, O.J., 2018. Additional increased effects of mannitol-temozolomide combined treatment on blood-brain

barrier permeability. Biochem. Biophys. Res. Commun. 497, 769-775. https://doi.org/10.1016/j.bbrc.2018.02.149

Christodoulou, E., Kechagia, I., Tzimas, S., Balafas, E., Kostomitsopoulos, N., Archontaki, H., Dokoumetzidis, A., Valsami, G., 2015. Serum and tissue pharmacokinetics of silibinin after per os and i.v. administration to mice as a HP-β-CD lyophilized product. Int. J. Pharm. 493, 366-373. https://doi.org/10.1016/j.ijpharm.2015.07.060

Colombo, G., Bortolotti, F., Chiapponi, V., Buttini, F., Sonvico, F., Invernizzi, R., Quaglia, F., Danesino, C., Pagella, F., Russo, P., Bettini, R., Colombo, P., Rossi, A., 2016. Nasal powders of thalidomide for local treatment of nose bleeding in persons affected by hereditary hemorrhagic telangiectasia. Int. J. Pharm. 514, 229–237. https://doi.org/10.1016/j.ijpharm.2016.07.002

Cuello, A.C., 2017. Early and late CNS inflammation in Alzheimer's disease: two extremes of a continuum?. Trends Pharmacol. Sci. 38, 956-966. https://doi.org/10.1016/j.tips.2017.07.005

Deardorff, W.J., Grossberg, G.T., 2017. Targeting neuroinflammation in Alzheimer's disease: evidence for NSAIDs and novel therapeutics. Expert Rev. Neurother. 17, 17-32. https://doi.org/10.1080/14737175.2016.1200972

Dhuria, S.V., Hanson, L.R., Frey, W.H., 2010. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. J. Pharm. Sci. 99, 1654-1673. https://doi.org/10.1002/jps.21924

Eriksen, J.L., Sagi, S.A., Smith, T.E., Weggen, S., Das, P., McLendon, D.C., Ozols, V.V., Jessing, K.W., Zavitz, K.H., Koo, E.H., Golde, T.E., 2003. NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo. J. Clin. Invest. 112, 440-449. https://doi.org/10.1172/JCI18162

European Medicines Agency: CPMP/ICH/381/95 - Note for Guidance on Validation of Analytical Procedures: Text and Methodology. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf (accessed 12 March 2021).

Giuliani, A., Balducci, A.G., Zironi, E., Colombo, G., Bortolotti, F., Lorenzini, L., Galligioni, V., Pagliuca, G., Scagliarini, A., Calzà, L., Sonvico, F., 2018. In vivo nose-to-brain delivery of the hydrophilic antiviral ribavirin by microparticle agglomerates. Drug Deliv. 25, 376-387. https://doi.org/10.1080/10717544.2018.1428242

Hershey, L.A., Lipton, R.B., 2019. Naproxen for presymptomatic Alzheimer disease: Is this the end, or shall we try again?. Neurology. 92, 829-830. https://doi.org/10.1212/WNL.0000000000007233

Holbrook, A.J., Tustison, N.J., Marquez, F., Roberts, J., Yassa, M.A., Gillen, D.L., 2020. Alzheimer's Disease Neuroimaging Initiative, Anterolateral entorhinal cortex thickness as a new biomarker for early detection of Alzheimer's disease. Alzheimers Dement. (Amst). 12, e12068. https://doi.org/10.1002/dad2.12068

in t' Veld, B.A., Ruitenberg, A., Hofman, A., Launer, L.J., van Duijn, C.M., Stijnen, T., Breteler, M.M., Stricker, B.H., 2001. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N. Engl. J. Med. 345, 1515-1521. https://doi.org/10.1056/NEJMoa010178

Inoue, D., Furubayashi, T., Tanaka, A., Sakane, T., Sugano, K., 2020. Effect of Cerebrospinal Fluid Circulation on Nose-to-Brain Direct Delivery and Distribution of Caffeine in Rats. Mol. Pharmaceutics. 17, 4067–4076. https://doi.org/10.1021/acs.molpharmaceut.0c00495

Jaturapatporn, D., Isaac, M.G., McCleery, J., Tabet, N., 2012. Aspirin, steroidal and non-steroidal anti-inflammatory drugs for the treatment of Alzheimer's disease. Cochrane Database Syst. Rev. 2, CD006378. https://doi.org/10.1002/14651858.CD006378.pub2

Kozlovskaya, L., Abou-Kaoud, M., Stepensky, D., 2014. Quantitative analysis of drug delivery to the brain via nasal route. J Control. Release. 189, 133-140. https://doi.org/10.1016/j.jconrel.2014.06.053

Lehrer, S., 2014. Nasal NSAIDs for Alzheimer's Disease. Am. J. Alzheimers Dis. Other Demen. 29, 401-403. https://doi.org/10.1177/1533317513518658

Lochhead, J.J., Thorne, R.G., 2012. Intranasal delivery of biologics to the central nervous system. Adv. Drug Deliv. Rev. 64, 614-628. https://doi.org/10.1016/j.addr.2011.11.002

McGeer, P.L., Guo, J.P., Lee, M., Kennedy, K., McGeer, E.G., 2018. Alzheimer's Disease Can Be Spared by Nonsteroidal Anti-Inflammatory Drugs. J. Alzheimers Dis. 62, 1219-1222. https://doi.org/10.3233/JAD-170706

McGeer, P.L., Rogers, J., McGeer, E.G., 2016. Inflammation, Antiinflammatory Agents, and Alzheimer's Disease: The Last 22 Years. J. Alzheimers Dis. 54, 853-857. https://doi.org/10.3233/JAD-160488

McGeer, P.L., Schulzer, M., McGeer, E.G., 1996. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. Neurology. 47, 425-432. https://doi.org/10.1212/wnl.47.2.425

Meister, S., Zlatev, I., Stab, J., Docter, D., Baches, S., Stauber, R.H., Deutsch, M., Schmidt, R., Ropele, S., Windisch, M., Langer, K., Wagner, S., von Briesen, H., Weggen, S., Pietrzik, C.U., 2013. Nanoparticulate flurbiprofen reduces amyloid-β 42 generation in an in vitro blood–brain barrier model. Alzheimers Res. Ther. 5, 51. https://doi.org/10.1186/alzrt225

Parepally, J.M.R., Mandula, H., Smith, Q.R., 2006. Brain Uptake of Nonsteroidal Anti-Inflammatory Drugs: Ibuprofen, Flurbiprofen, and Indomethacin. Pharm. Res. 23, 873–881. https://doi.org/10.1007/s11095-006-9905-5

Pozzoli, M., Traini, D., Young, P.M., Sukkar, M.B., Sonvico, F., 2017. Development of a Soluplus budesonide freeze-dried powder for nasal drug delivery. Drug Dev. Ind. Pharm. 43, 1510-1518. https://doi.org/10.1080/03639045.2017.1321659

Raffin, R.P., Colombo, P., Sonvico, F., Polleto, F.S., Colombo, G., Rossi, A., Pohlmann, A.R., Guterres, S.S., 2007. Soft agglomerates of pantoprazole gastro-resistant microparticles for oral administration and intestinal release. J. Drug Del. Sci. Tec. 17, 407–413. https://doi.org/10.1016/S1773-2247(07)50081-X

Rassu, G., Ferraro, L., Pavan, B., Giunchedi, P., Gavini, E., Dalpiaz, A., 2018. The Role of Combined Penetration Enhancers in Nasal Microspheres on In Vivo Drug Bioavailability. Pharmaceutics. 10, 206. https://doi.org/10.3390/pharmaceutics10040206

Rivers-Auty, J., Mather, A.E., Peters, R., Lawrence, C.B., Brough, D., 2020. Anti-inflammatories in Alzheimer's disease—potential therapy or spurious correlate?. Brain Communications. 2, fcaa109. https://doi.org/10.1093/braincomms/fcaa109

Russo, P., Buttini, F., Sonvico, F., Bettini, R., Massimo, G., Sacchetti, C., Colombo, P., Santi, P., 2004. Chimeral agglomerates of microparticles for the administration of caffeine nasal powders. J. Drug Deliv. Sci. Tec. 14, 449-454. https://doi.org/10.1016/S1773-2247(04)50083-7

Russo, P., Sacchetti, C., Pasquali, I., Bettini, R., Massimo, G., Colombo, P., Rossi, A., 2006. Primary microparticles and agglomerates of morphine for nasal insufflation. J. Pharm. Sci. 95, 2553-2561. https://doi.org/10.1002/jps.20604

Suico, J.G., Hövelmann, U., Zhang, S., Shen, T., Bergman, B., Sherr, J., Zijlstra, E., Frier, B.M., Plum-Mörsche,l L., 2020. Glucagon Administration by Nasal and Intramuscular Routes in Adults With Type 1 Diabetes During Insulin-Induced Hypoglycaemia: A Randomised, Open-Label, Crossover Study. Diabetes Ther. 11, 1591–1603. https://doi.org/10.1007/s13300-020-00845-7

Szpunar, G.J., Albert, K.S., Bole, G.G., Dreyfus, J.N., Lockwood, G.F., Wagner, J.G., 1987. Pharmacokinetics of flurbiprofen in man. I. Area/dose relationships. Biopharm. Drug Dispos. 8, 273-283. https://doi.org/10.1002/bdd.2510080308

Tanaka, A., Furubayashi, T., Matsushita, A., Inoue, D., Kimura, S., Katsumi, H., Sakane, T., Yamamoto, A., 2016. Nasal Absorption of Macromolecules from Powder Formulations and Effects of Sodium Carboxymethyl Cellulose on Their Absorption. PLoS One. 11, e0159150. https://doi.org/10.1371/journal.pone.0159150

Tiozzo Fasiolo, L., Manniello, M.D., Bortolotti, F., Buttini, F., Rossi, A., Sonvico, F., Colombo, P., Valsami, G., Colombo, G., Russo, P., 2019. Anti-inflammatory flurbiprofen nasal powders for nose-to-brain delivery in Alzheimer's disease. J. Drug Target. 27, 984-994. https://doi.org/10.1080/1061186X.2019.1574300

Tiozzo Fasiolo, L., Manniello, M.D., Tratta, E., Buttini, F., Rossi, A., Sonvico, F., Bortolotti, F., Russo, P., Colombo, G., 2018. Opportunity and challenges of nasal powders: Drug formulation and delivery. Eur. J. Pharm. Sci. 113, 2-17. https://doi.org/10.1016/j.ejps.2017.09.027

Wong, L.R., Ho, P.C., 2018. Role of serum albumin as a nanoparticulate carrier for nose-to-brain delivery of R-flurbiprofen: implications for the treatment of Alzheimer's disease. J. Pharm. Pharmacol. 70, 59-69. https://doi.org/10.1111/jphp.12836

Xi, J., Kim, J.W., Si, X.A., Corley, R.A., Zhou, Y., 2016. Modeling of inertial deposition in scaled models of rat and human nasal airways: Towards in vitro regional dosimetry in small animals. J. Aerosol Sci. 99, 78-93. https://doi.org/10.1016/j.jaerosci.2016.01.013

SUPPLEMENTARY MATERIAL

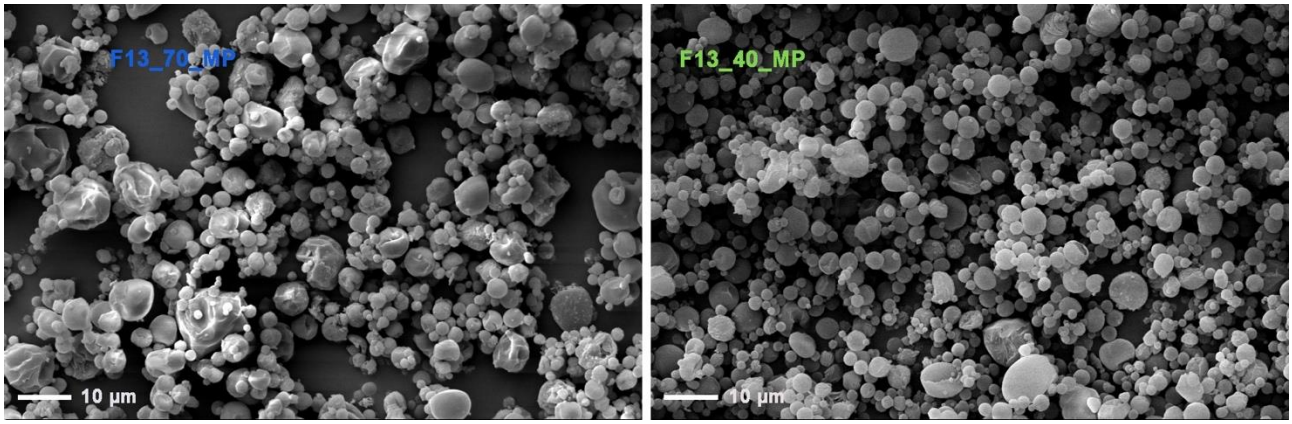


Figure S1. SEM micrographs (2500x; size bar: 10 µm). From left to right: FB-COONa microparticles spray-dried at 70 °C (F13_70_MP); FB-COONa microparticles spray-dried at 40 °C (F13_40_MP).

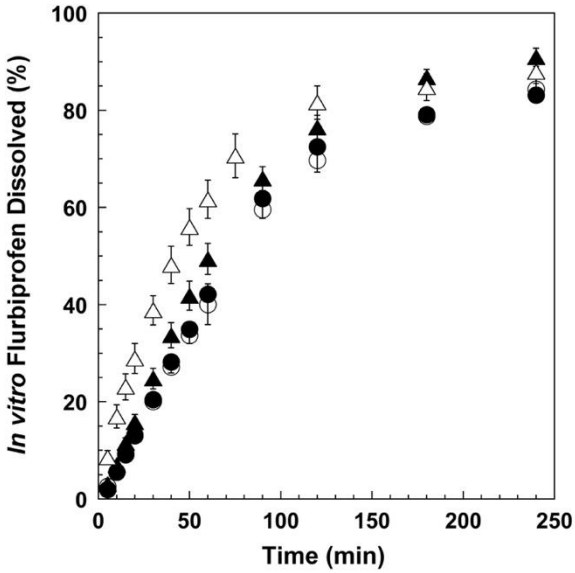


Figure S2. Flurbiprofen dissolution/transport across a regenerated cellulose membrane from soft pellets (triangle) of F13_40_MP (white) and F13_70_MP (black) *vs.* the primary microparticles (circle) F13_40_MP (white) and F13_70_MP (black) (mean ± SEM, n=3). Data for the microparticles have been re-elaborated from Tiozzo Fasiolo L. et al. Tiozzo Fasiolo et al., 2019).

Prof. Gaia Colombo
Dept. of Life Sciences and Biotechnology
University of Ferrara
Via Fossato di Mortara 17/19
I-Ferrara, 44121

Tel. +39 0532 455909
clmgai@unife.it

Prof. Carmen Alvarez-Lorenzo
Prof. Abdul Basit
Prof. Leena Peltonen
Prof. Raymond Schiffelers

Associate Editors for Europe
International Journal of Pharmaceutics

Ferrara, March 12th, 2021

Object: **Research Article submission**

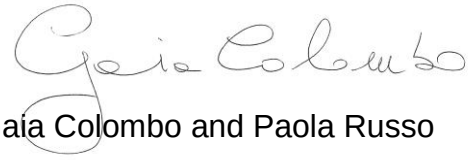
Dear professors,

on behalf of all co-authors, prof. Russo and I are pleased to submit to your attention our manuscript entitled “Flurbiprofen Sodium Microparticles and Soft Pellets for Nose-to-Brain Delivery: Serum and Brain Pharmacokinetics in Rats after Nasal Insufflation” (Original Research Article).

The work is original as it shows for the first time that flurbiprofen enters the brain directly by means of properly formulated nasal powders, whose efficiency in terms of drug brain disposition outperformed a conventional flurbiprofen solution given intranasally or intravenously. The significance of flurbiprofen nose-to-brain delivery to address early Alzheimer’s disease (AD), relies on the fact that remarkable drug concentrations were measured in the olfactory bulb, connected with the entorhinal cortex from where AD initiates. Moreover, we discovered that nasal powder technology (microparticles or soft pellets) diversified flurbiprofen absorption into serum and brain. We look forward to our manuscript being considered for publication in International Journal of Pharmaceutics.

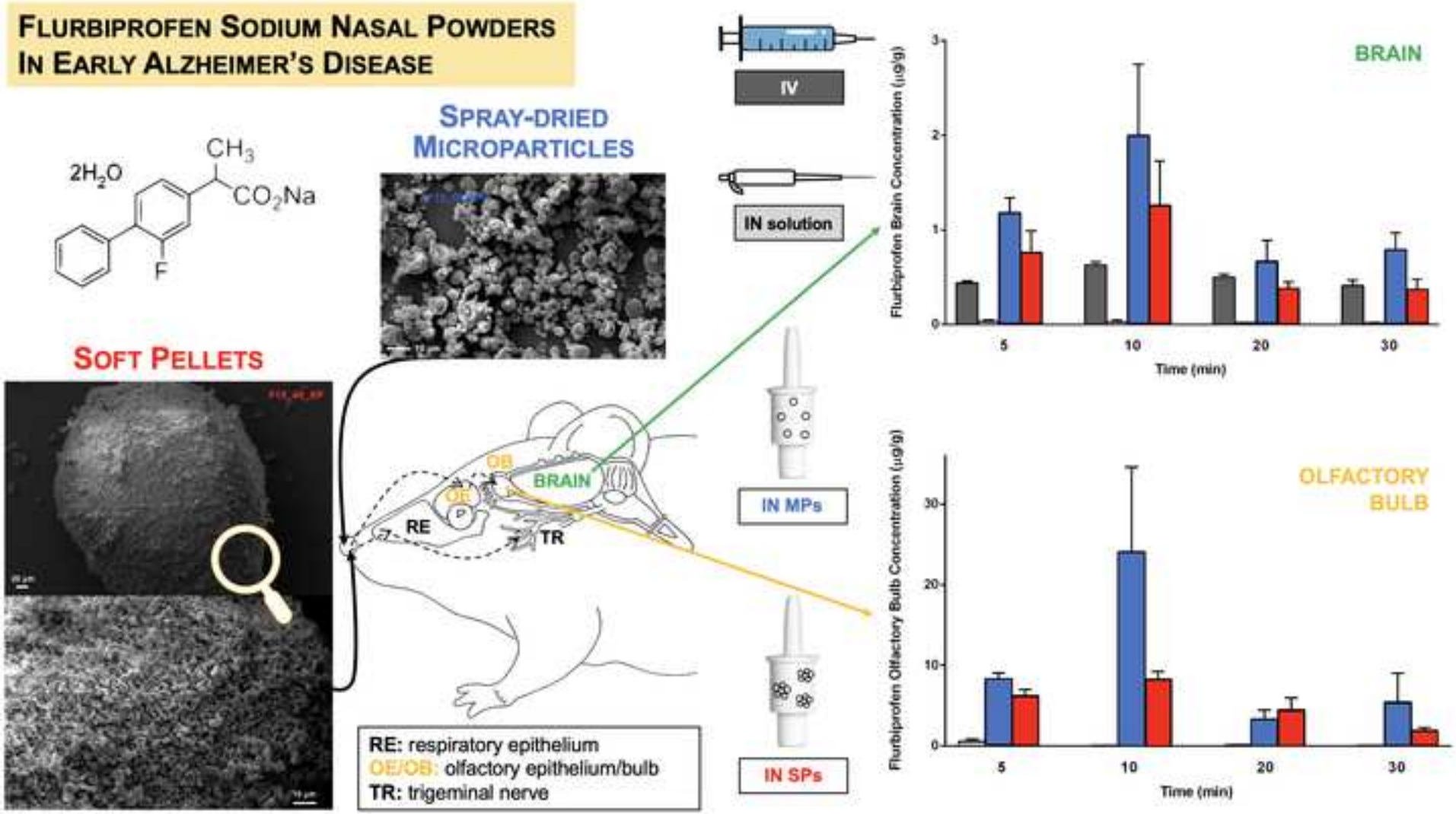
Thank you.

Sincerely yours,



Gaia Colombo and Paola Russo

Graphical Abstract (for review)



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Yours faithfully,
Gaia Colombo

Research article

Flurbiprofen Sodium Microparticles and Soft Pellets for Nose-to-Brain Delivery: Serum and Brain Pharmacokinetics in Rats after Nasal Insufflation

HIGHLIGHTS

- Flurbiprofen enters brain directly by nasal microparticle or soft pellet powders.
- Drug brain disposition by powders outperformed the nasal solution.
- The flurbiprofen nasal powder technology diversified serum and brain absorption.
- Flurbiprofen microparticles provided remarkable levels in olfactory bulb.
- Olfactory bulb connects with entorhinal cortex where Alzheimer’s disease initiates.

TABLES

Table I. *Soft pellets of flurbiprofen sodium (FB-COONa) and excipient spray-dried microparticles mixtures (size range 106-500 µm).*

FB-COONa microparticles	FB-COONa/excipient ratio	FB-COOH content (% w/w)
F13_70_MP	50:50	34.6 ± 2.3
F13_40_MP	50:50	35.0 ± 0.7

Table II. *Nasal insufflation in rats from UDS powder device of flurbiprofen sodium spray-dried microparticles (F13_70_MP) and soft pellets of flurbiprofen sodium spray-dried microparticles with excipient microparticles (F13_40_SP). Data are reported as mean ± standard deviation (n≥13).*

Nasal Powder	**Powder Loaded (mg)**	**Powder Emitted (mg)**	**FB-COOH Emitted (mg)**
F13_70_MP (Microparticles)	12.9 ± 0.9 (10.4 mg FB-COOH)	8.4 ± 1.2 (65%)	6.7 ± 1.0
F13_40_SP (Soft pellets)	14.6 ± 0.7 (5.1 mg FB-COOH)	11.9 ± 1.1 (83%)	4.2 ± 0.4

Table III. Area Under the Curve (AUC) calculated in serum and in brain, Drug Targeting Efficiency (DTE) and Direct Transport Percentage (DTP) of flurbiprofen for intranasal drug powders, namely F13_70 microparticles and F13_40 soft pellets, and drug solution compared to IV administration. AUC data are expressed as mean ± SEM.

Treatment	**AUC_{0-t} serum** (μg ml^{-1} min)	**AUC_{0-t} brain** (μg g^{-1} min)	**DTE** (%)	**DTP** (%)
IV solution	3528.5 ± 184.8	13.97 ± 0.50	-	-
F13_70_MP	1748.4 ± 171.5	31.56 ± 6.14	456	78
F13_40_SP	1912.0 ± 113.2	18.99 ± 3.79	251	60
IN solution	226.3 ± 22.2	0.65 ± 0.18	72	-39

Figure 1

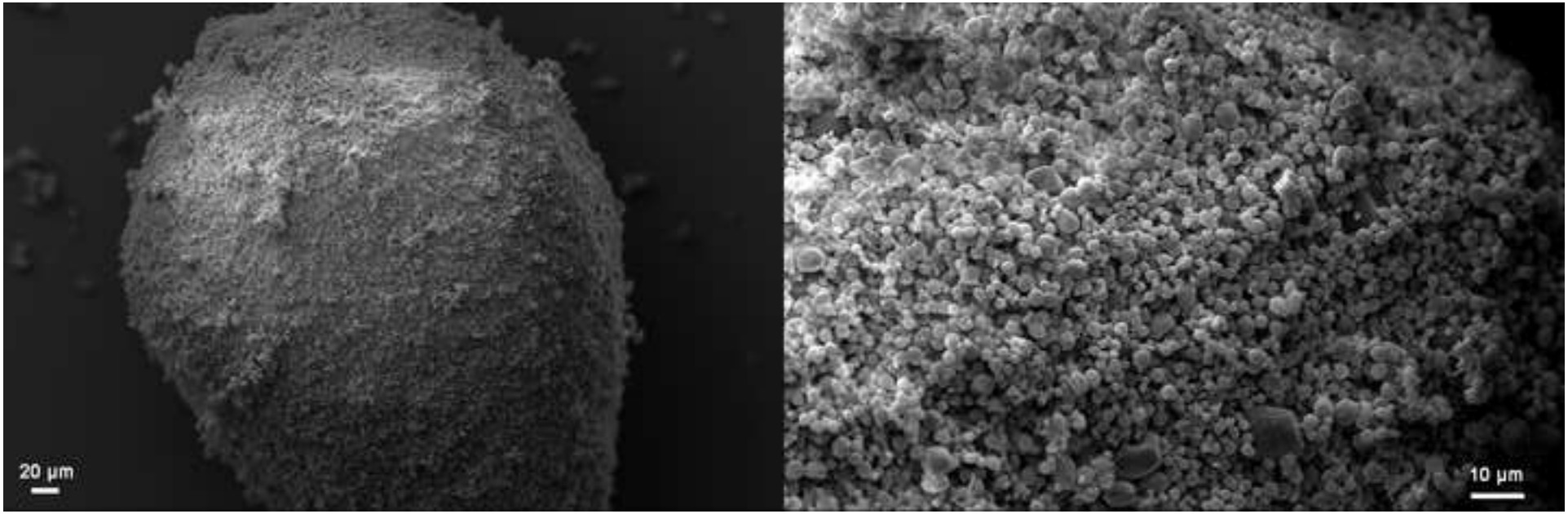


Figure 2

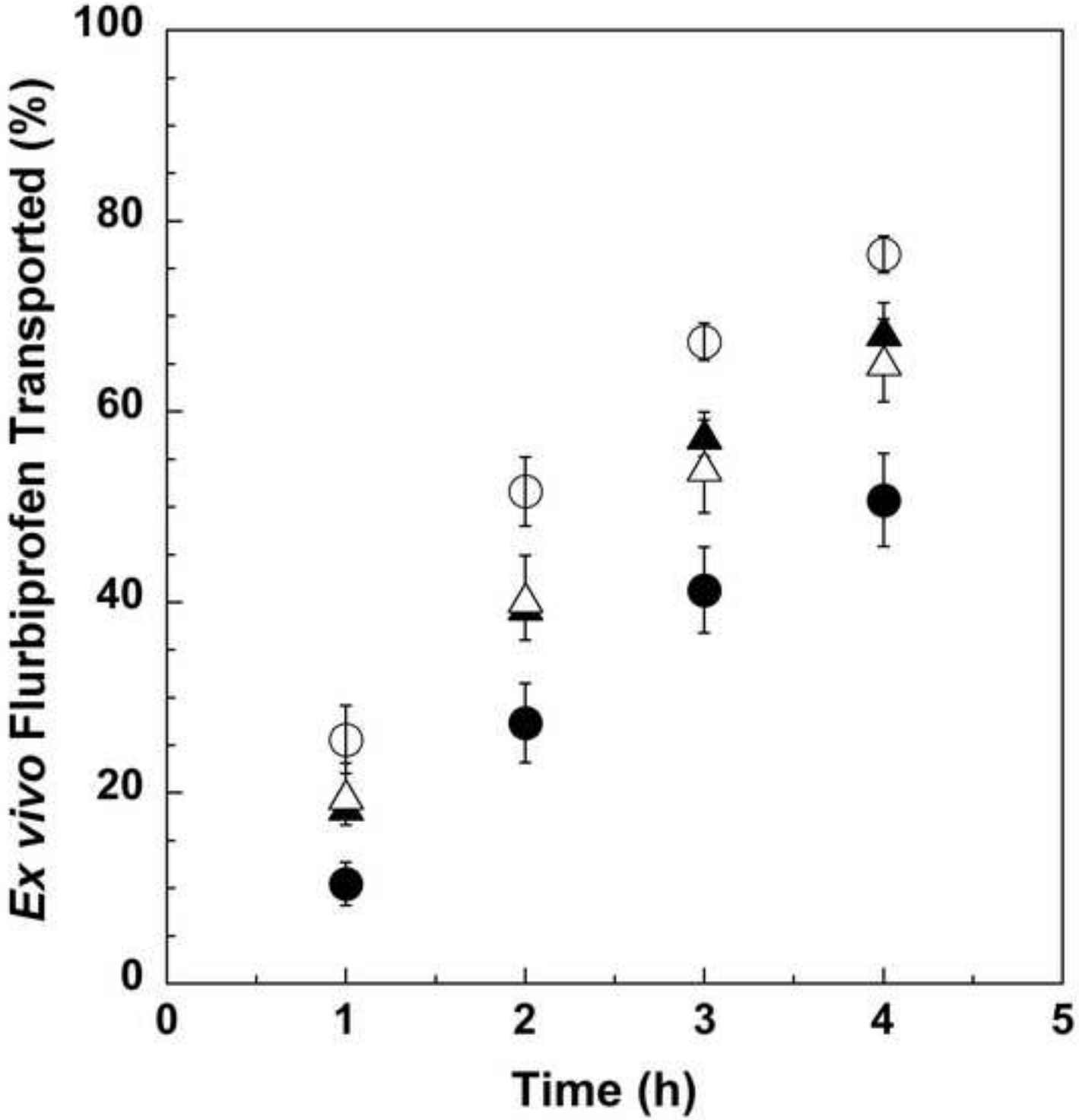

Ex vivo Flurbiprofen Transported (%)
100
80
60
40
20
0
0
1
2
3
4
5
Time (h)

Figure 3

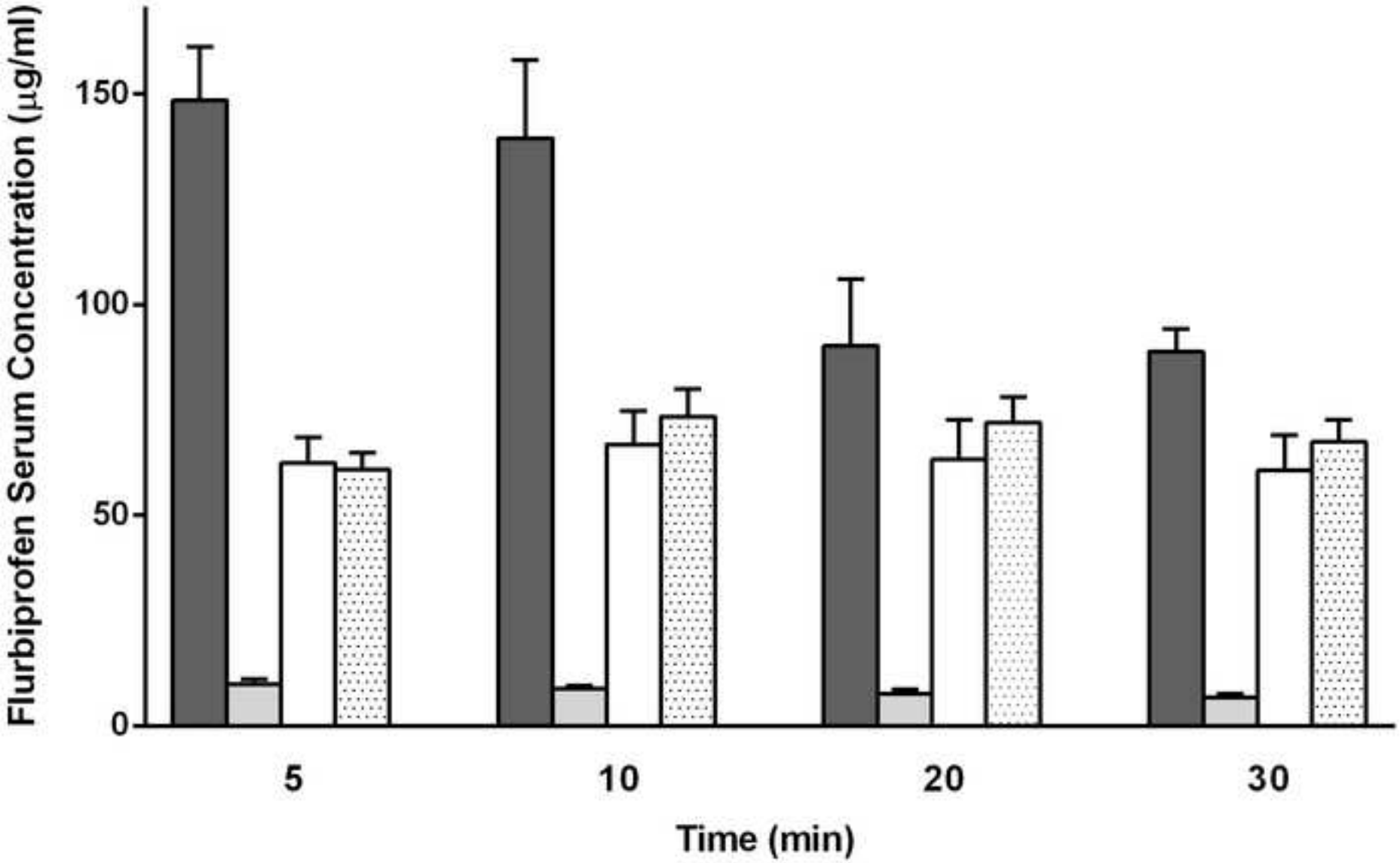

Flurbiprofen Serum Concentration (μg/ml)
150
100
50
0
5
10
20
30
Time (min)

Figure 4, panel A

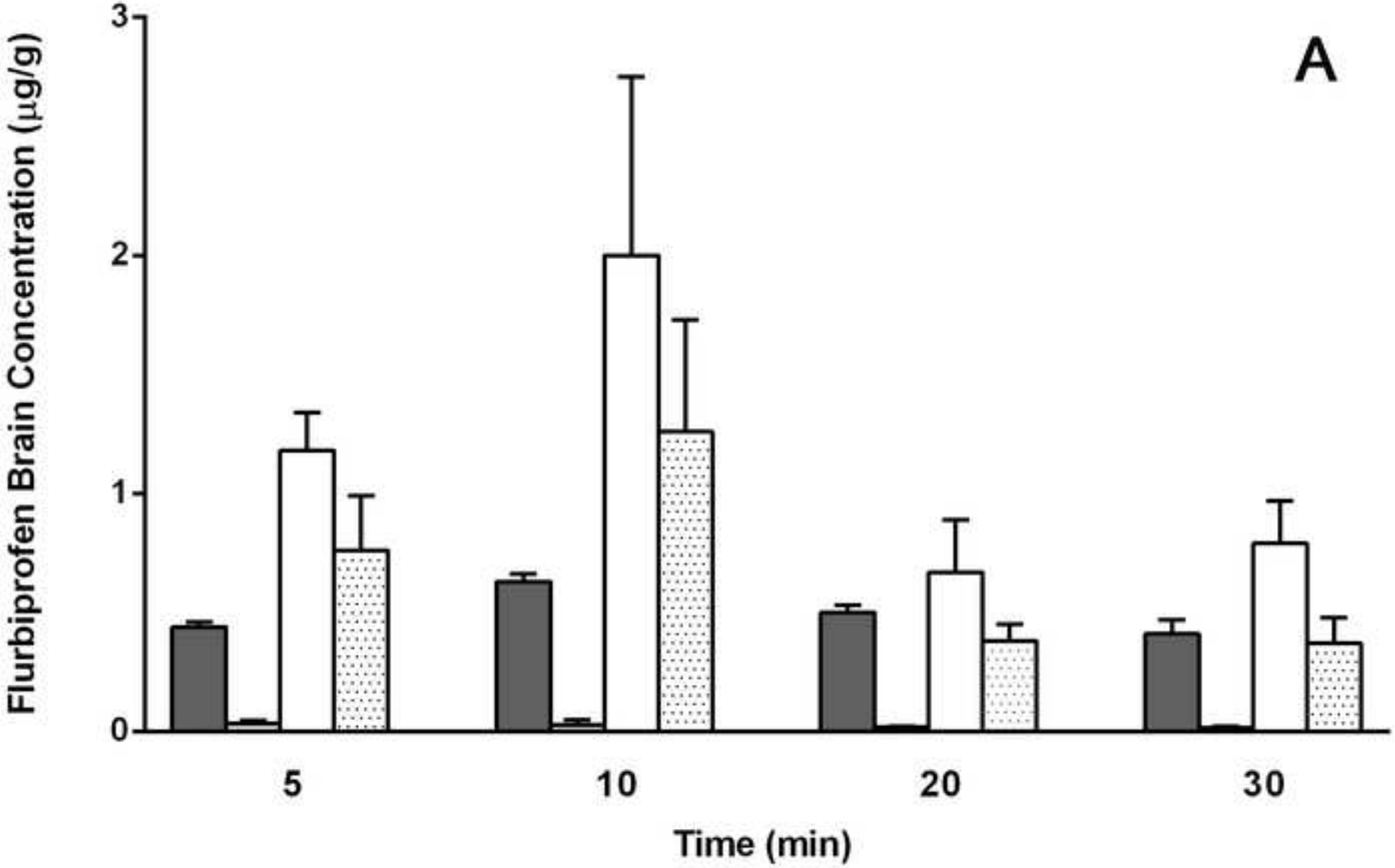


Figure 4, panel B

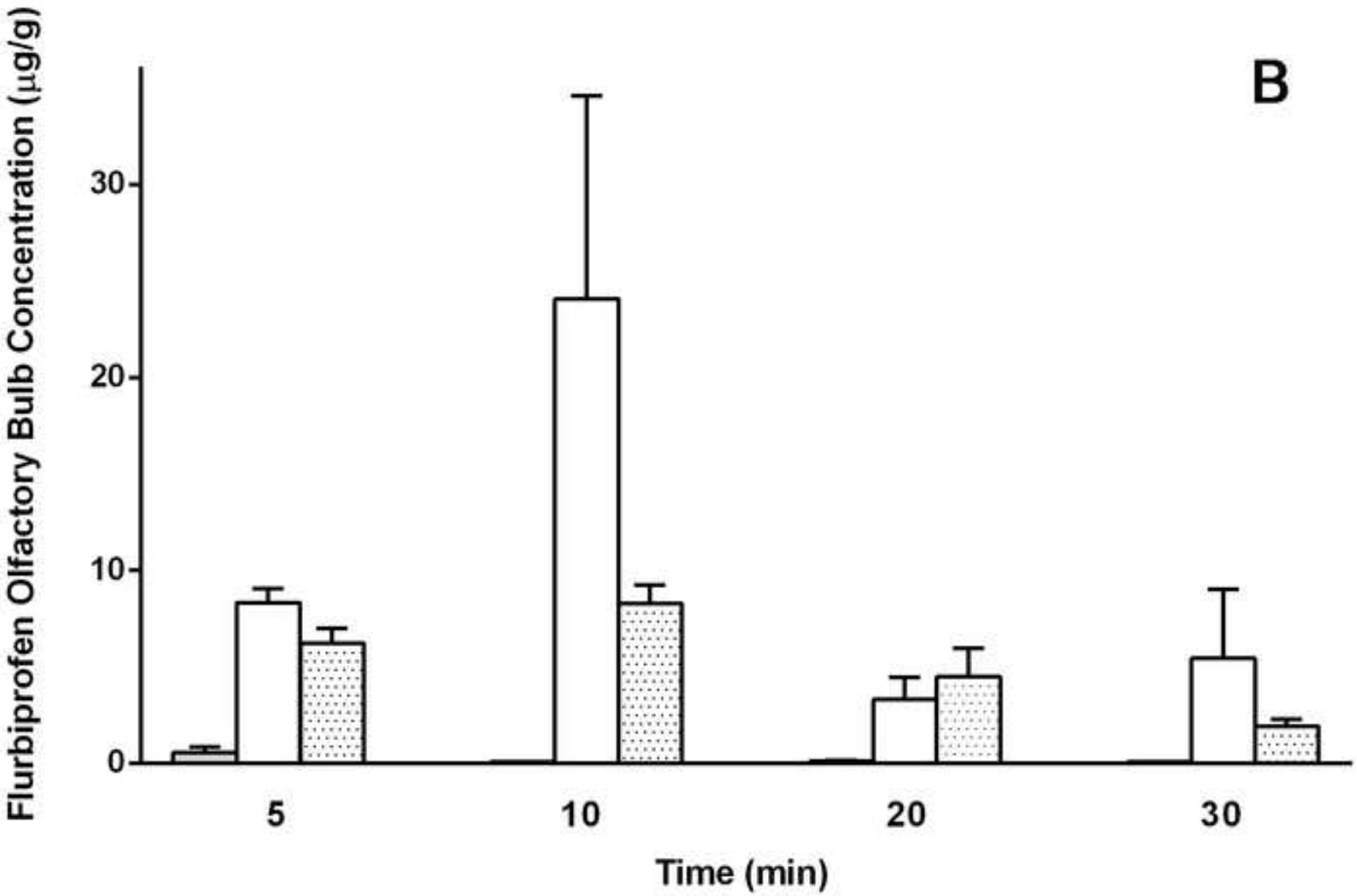


FIGURE CAPTIONS

Figure 1. SEM micrographs of (from left to right): F13_40_SP soft pellet (500x) and a detail of its surface (2000x).

Figure 2. Flurbiprofen transport across rabbit nasal mucosa from soft pellets (triangle) of F13_40_MP (white) and F13_70_MP (black) *vs.* the primary microparticles (circle) F13_40_MP (white) and F13_70_MP (black) (mean ± SEM, n≥5). Data for the microparticles have been re-elaborated from Tiozzo Fasiolo et al. (2019).

Figure 3. Flurbiprofen serum concentration *vs.* time after intravenous (IV; dark grey) and intranasal (IN) administrations: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white). Data are expressed as mean ± SEM (n≥3).

Figure 4. Flurbiprofen concentration in brain (panel A) and olfactory bulb (panel B) after intranasal (IN) administration of: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white) *vs.* intravenous injection (IV; dark grey). Flurbiprofen levels in olfactory bulb were not measured for the IV treatment. Data are expressed as mean ± SEM (n≥3).

Figure S1


Click here to access/download
Supplementary Material
Figure S1.jpg

Figure S2


Click here to access/download
Supplementary Material
Figure S2.jpg

Captions of supplementary figures