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

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Abstract:	<p>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.</p> <p>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.</p> <p>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</p>

	cortex, from where AD initiates, makes promising flurbiprofen sodium administration as nasal powder.
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1 *Research Article*

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

4

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35 **ABSTRACT**

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

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

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48 Nevertheless, efficiency of powder delivery by the device, measured as emitted fraction,
49 was superior with soft pellets than microparticles, due to their coarse size. Both nasal
50 powders resulted into rapid flurbiprofen absorption. Absolute bioavailability was 33% and
51 58% for microparticles and pellets, respectively. Compared to intravenous flurbiprofen, the
52 microparticles were more efficient than soft pellets at enhancing direct drug transport to
53 CNS. Direct Transport Percentage index evidenced that more than 60% of the intranasal
54 dose reached the brain via direct nose-to-brain transport for both powders. Moreover,
55 remarkable drug concentrations were measured in the olfactory bulb after microparticle
56 delivery. Bulb connection with the entorhinal cortex, from where AD initiates, makes
57 promising flurbiprofen sodium administration as nasal powder.

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60 **KEYWORDS**

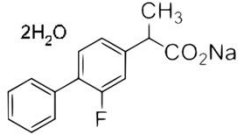
61 Alzheimer's disease, flurbiprofen sodium, nasal powder, nose-to-brain transport,
62 microparticle, soft pellet.

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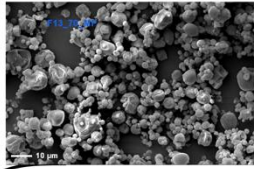
64 **GRAPHICAL ABSTRACT**

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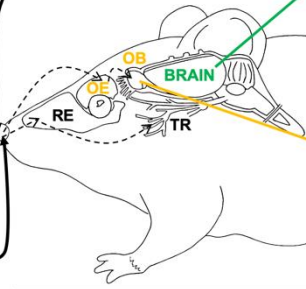
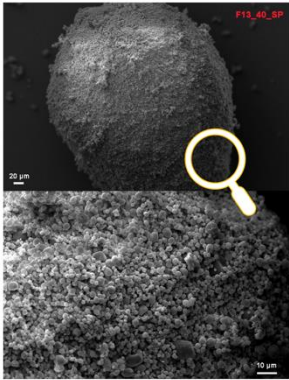
**FLURBIPROFEN SODIUM NASAL POWDERS
IN EARLY ALZHEIMER'S DISEASE**



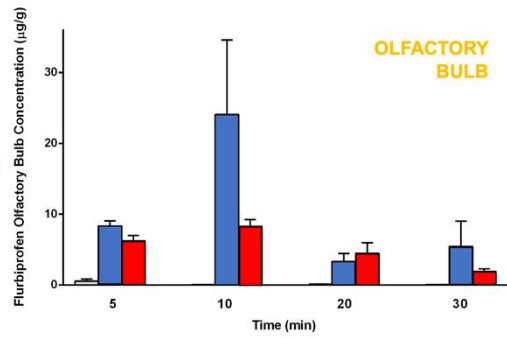
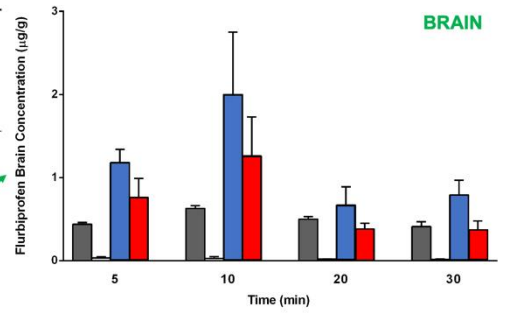
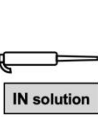
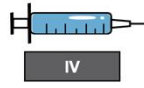
**SPRAY-DRIED
MICROPARTICLES**



SOFT PELLETS



RE: respiratory epithelium
OE/OB: olfactory epithelium/bulb
TR: trigeminal nerve



87 **LIST OF ABBREVIATIONS**

88	AD	Alzheimer's Disease
89	AUC	Area Under the Curve
90	BBB	Blood Brain Barrier
91	C _{max}	Maximum Concentration (peak)
92	CNS	Central Nervous System
93	DTE	Drug Targeting Efficiency
94	DTP	Direct Transport Percentage
95	FB-COOH	Flurbiprofen
96	FB-COONa	Flurbiprofen sodium
97	FDA	Food and Drug Administration
98	FLD	Fluorescence Detection
99	HPLC	High-performance liquid chromatography
100	IN	Intranasal
101	IV	Intravenous
102	LOD	Limit of Detection
103	LOQ	Limit of Quantification
104	MP	Microparticle/s
105	MW	Molecular Weight
106	NCA	Non-Compartmental Analysis
107	NSAIDs	Non Steroidal Anti-Inflammatory Drugs
108	PBS	Phosphate Buffered Saline
109	PK	Pharmacokinetics
110	RSD	Relative Standard Deviation
111	SEM	Standard Error of the Mean
112	SP	Soft Pellet/s
113	t _{max}	Time-to-peak of maximum concentration
114	UDS	Unidose Powder System
115		

116 1. INTRODUCTION

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

127 Recently, the role of inflammation in the AD pathology has been focused, postulating the
128 existence of an early and a late inflammation in the CNS (Cuello, 2017). The early
129 neuroinflammation revamped the role of a preventive therapeutic action for AD, that may
130 be more effective than treating the late inflammation phase (Deardorff and Grossberg,
131 2017; McGeer et al., 2016). Therefore, the anti-inflammatory activity of flurbiprofen is worth
132 being exploited to delay the onset of the disease, provided that its access to brain is
133 enhanced, while limiting systemic exposure (Hershey and Lipton, 2019; Rivers-Auty et al.,
134 2020).

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

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

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

150 *in vivo* transmucosal transport were obtained with the use of flurbiprofen sodium salt
151 microparticles. Racemic flurbiprofen was chosen, since both enantiomers are of interest in
152 the early AD prevention (Meister et al., 2013). In fact, the S-enantiomer has anti-
153 inflammatory activity, whereas the R one inhibits the gamma secretase enzyme, involved
154 in amyloid plaques deposition (Eriksen et al., 2003; Wong and Ho, 2018).
155 The aim of the present study was to investigate the insufflation, deposition, dissolution,
156 transmucosal permeation, and *in vivo* transport to rat brain of flurbiprofen sodium nasal
157 powders combined in an active delivery device. To gain an advantage in powder metering
158 and deposition into the nasal cavity, agglomerated microparticles in the form of soft pellets
159 were studied in comparison with the primary microparticulate powders. Thus, two nasal
160 powders were tested, i.e., spray-dried flurbiprofen sodium microparticles and soft pellets
161 thereof. Following insufflation of the powders into rat nasal cavity, flurbiprofen fraction
162 absorbed and brain disposition were assessed, determining drug concentration in serum,
163 olfactory bulb and total brain. For comparison, intravenous and intranasal solutions of
164 flurbiprofen sodium were administered as well.

165

166

167 **2. MATERIALS AND METHODS**

168 **2.1 Materials**

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

179

180 **2.2 Preparation of Flurbiprofen Formulations**

181 2.2.1 Drug solution for intravenous and nasal administration

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

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

190

191 2.2.2 Spray-dried microparticle powder

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

201

202 2.2.3 Agglomerated powder of spray-dried microparticles (soft pellets)

203 Since the sodium flurbiprofen microparticles manufactured with the Nano-spray dryer did
204 not spontaneously agglomerate, the soft pellets were prepared according to Balducci et al.
205 (2013). For the purpose, spray-dried excipient microparticles of mannitol and lecithin (ratio
206 92:8 w/w) were prepared by spray drying a 2% (w/v) total solid solution in
207 water:isopropanol (92:8 v/v) using the Nano B-90 spray dryer (Büchi, CH-Flawil) at an inlet
208 temperature of 40 °C.

209 The soft pellets were then prepared as follows: spray-dried drug microparticles were
210 manually and carefully mixed with spray-dried mannitol/lecithin microparticles (mass ratio
211 1:1). After assessing its homogeneous drug content, the microparticle blend was tumbled
212 in a 100 ml glass pan having deflected walls (DISA, IT-Sesto San Giovanni, MI). The pan
213 was fixed to the rotating arm of tablet friability tester at a 90° angle and rotated at 25
214 rpm/min for 40 min. The agglomerated powder obtained was manually sieved through a
215 500 μm sieve and collected on top of a 106 μm sieve. Thus, the soft pellets used for *in*
216 *vivo* administration had a size in the range 106-500 μm .

217

218 **2.3 Powder Dissolution and *Ex Vivo* Permeation**

219 *In vitro* dissolution and *ex vivo* permeation of flurbiprofen from the nasal powders were
220 determined with Franz-type vertical diffusion cells (0.58 cm²), using either a regenerated
221 cellulose membrane or freshly excised rabbit nasal mucosa as barrier. The nasal tissue
222 was extracted within 2 h from the animal's death from rabbit heads supplied by a local
223 slaughterhouse (Pola S.r.l., IT- Finale Emilia, MO). Equipment and experimental conditions
224 were according to our previous research work (Tiozzo Fasiolo et al., 2019). In order to
225 have the same drug amount with both formulations, the powder mass loaded into the cell's
226 donor chamber was about 5 mg for the drug microparticles and 11-12 mg for the soft
227 pellets. The volume of liquid (Phosphate Buffered Saline, PBS pH 7.4; KCl 0.2 g/l; NaCl 8
228 g/l; Na₂HPO₄ 1.15 g/l; KH₂PO₄ 0.2 g/l) added to wet the powder was the 100 µl,
229 independently of the powder mass.

230

231 **2.4 *In Vivo* Animal Experiments**

232 2.4.1 Nasal administration

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

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

243

244 2.4.2 Animals and housing conditions

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

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

262

263 2.4.3 Pharmacokinetic study protocol

264 Forty-eight 8-week-old Wistar-type rats (350 ± 50 g) were randomly divided in four groups.
265 The animals in each group received a different treatment, namely: a) **IV group** (12 rats)
266 received 0.3 ml of the 15 mg/ml drug solution intravenously as bolus through the tail artery
267 (FB-COOH dose: 4.5 mg); b) **IN solution group** (12 rats) received 0.02 ml of the 15 mg/ml
268 drug solution (FB-COOH dose: 0.3 mg); c) **IN microparticle powder group** (12 rats)
269 received intranasally an FB-COOH dose of 6.7 mg as spray-dried microparticles (coded
270 F13_70_MP); and d) **IN soft pellet powder group** (12 rats) received intranasally an FB-
271 COOH dose of 4.2 mg as soft pellets, obtained by agglomeration of flurbiprofen sodium
272 microparticles with mannitol-lecithin excipient microparticles (code F13_40_SP).
273 All treatments were carried out on anaesthetized rats. Anesthesia was induced by
274 intraperitoneal injection of ketamine (100 mg/kg) and xylazine (0.1 mg/kg). The intranasal
275 administration procedure was different for the solution and powders. In the first case, the
276 animals lay in supine position and 5 μ l fractions up to 20 μ l were instilled alternately into
277 both rat's nostrils, thus aiming to avoid nasopharynx deposition and respiratory distress.
278 The administration time was less than 1 min. For powder administration, the rat lay down
279 on the right side, making the left nostril accessible. Only the left nasal cavity was used for
280 powder insufflation. The tip of the nasal insufflator was inserted through the nostril for a
281 depth of 1-2 mm. The pump was actuated and the powder was emitted in one shot.
282 Immediately after use, the device was re-weighed to determine the quantity of powder
283 emitted and calculate the actual dose administered.
284 The time points of interest for measuring flurbiprofen levels in the brain were set at 5, 10,
285 20 and 30 min after treatment. For the purpose, the rats in each treatment group were

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

289 Blood samples were also taken via puncture of the lateral vesicular vein at all specified
290 time points until the animal sacrifice. Blood samples were collected in non-heparinized
291 Eppendorf tubes and immediately centrifuged to separate serum. Serum and brain
292 samples were frozen and stored at $-70\text{ }^{\circ}\text{C}$ until extraction and HPLC analysis.

293

294 **2.5 Flurbiprofen Extraction from Biological Samples**

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

297

298 2.5.1 Flurbiprofen extraction from rat serum

299 0.5 ml of ibuprofen solution in acetonitrile (0.7 mg/ml) and 0.05 ml of methanol were added
300 to 0.25 ml of serum sample and vortexed for 15 sec. After centrifugation (10 min, 7500
301 rpm, $20\text{ }^{\circ}\text{C}$) to precipitate the plasma proteins, the clear supernatant was analyzed to
302 quantify flurbiprofen as such or after dilution with blank serum, when flurbiprofen
303 concentration in serum exceeded the linearity range of the analytical method. The drug
304 extraction efficiency from serum samples was assessed in samples containing flurbiprofen
305 concentrations ranging from 5 to 1260 ng/ml. Drug recovery was 100% in the range 90-
306 1260 ng/ml flurbiprofen concentration in serum samples.

307

308 2.5.2 Flurbiprofen extraction from rat brain

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

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

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

329

330 **2.6 HPLC-FLD Method for Flurbiprofen Quantification in Biological Samples**

331 Flurbiprofen in biological samples was quantified by reverse-phase HPLC with
332 fluorescence detection (HPLC-FLD; Shimadzu, JP-Kyoto). Isocratic elution was carried out
333 with a NaH₂PO₄ 20 mM:CH₃CN (40:60) mobile phase (pH 3.0 ± 0.1) at 30 °C. The
334 detection wavelength was set at 254 nm and 308 nm for excitation and emission,
335 respectively. The column was a ZORBAX Eclipse XDB (C18, 5 µm, 4.6 x 150 mm; Agilent,
336 Santa Clara, CA, USA). The flow rate was 1 ml/min and injection volume 20 µl. In these
337 conditions, the retention time of flurbiprofen was 3.9 min, while the internal standard
338 (ibuprofen) was eluted at 5.1 min. The method was developed in-house and validated with
339 respect to linearity, repeatability, matrix effect, limit of quantification (LOQ) and limit of
340 detection (LOD).

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

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

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

360

361 **2.7 Non-Compartmental PK Analysis**

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

373

$$374 \frac{AUC_{0-t (IN)} \times Dose_{(IV)}}{AUC_{0-t (IV)} \times Dose_{(IN)}} \quad \text{Eq. 1}$$

375

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

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

381

382 **2.8 Statistical Analysis**

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

386

387 3. RESULTS AND DISCUSSION

388 3.1 Flurbiprofen Sodium Nasal Powders

389 3.1.1 Spray-dried microparticles and soft pellets

390 Flurbiprofen sodium spray-dried microparticles for nasal insufflation have been described
391 in a previous paper (Tiozzo Fasiolo et al., 2019). A dry powder product for nasal deposition
392 is a combination of the drug formulation with a nasal insufflator. Both components
393 contribute to the efficiency of the delivery during insufflation. In this work, the nasal
394 formulation of flurbiprofen sodium spray-dried microparticles has been studied in
395 comparison with the same microparticles agglomerated in soft pellets. Soft pellets have
396 proved to be suitable for combination with insufflator devices for nasal powder (Balducci et
397 al., 2013; Giuliani et al., 2018; Russo et al., 2006), since their free-flowing characteristics
398 facilitate the dose metering and emission. During insufflation into the nose, the air flow
399 turbulence applied by the device breaks the soft pellets into fragments (Giuliani et al.,
400 2018). These fragments, composed of several microparticles, have suitable size for nasal
401 deposition by impaction on the epithelium; at the same time, they mitigate the risk of lung
402 entrance (Russo et al., 2004). After deposition, in contact with the nose mucosal fluid, the
403 soft pellet fragments disaggregate restoring the primary microparticles that quickly dissolve
404 (Balducci et al., 2013; Raffin et al., 2007; Russo et al., 2004; Russo et al., 2006).
405 Among the microparticulate powders described in the previous paper (Tiozzo Fasiolo et
406 al., 2019), two powders of flurbiprofen sodium spray-dried at different temperature with the
407 Nano spray dryer B-90 (coded F13_70_MP and F13_40_MP), were selected for the *in vivo*
408 animal study (Fig. S1 in Supplementary Material). Unfortunately, these flurbiprofen
409 powders did not spontaneously agglomerate. As shown by Giuliani et al. (2018), mannitol
410 microparticles containing lecithin as binding agent, here made by means of the Nano B-90-
411 spray dryer, easily agglomerated. Therefore, 1:1 blends of flurbiprofen sodium (FB-
412 COONa) spray-dried microparticles and mannitol/lecithin spray-dried microparticles were
413 prepared. By tumbling these mixtures, soft pellets containing flurbiprofen sodium were
414 constructed in size range 106-500 μm .

415
416 *Table I. Soft pellets of flurbiprofen sodium (FB-COONa) and excipient spray-dried microparticles*
417 *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 \pm 2.3
F13_40_MP	50:50	35.0 \pm 0.7

418

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

424

425

426

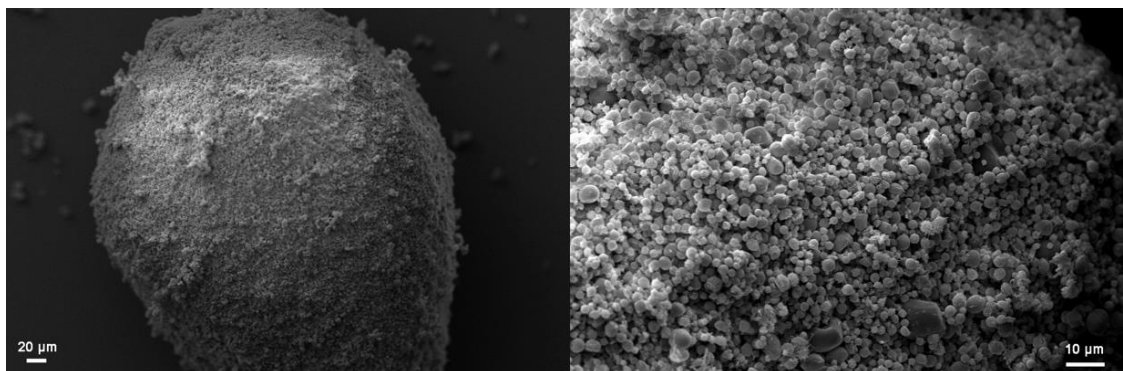
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432 *Figure 1. SEM micrographs of (from left to right): F13_40_SP soft pellet (500x) and a detail of its*
433 *surface (2000x).*

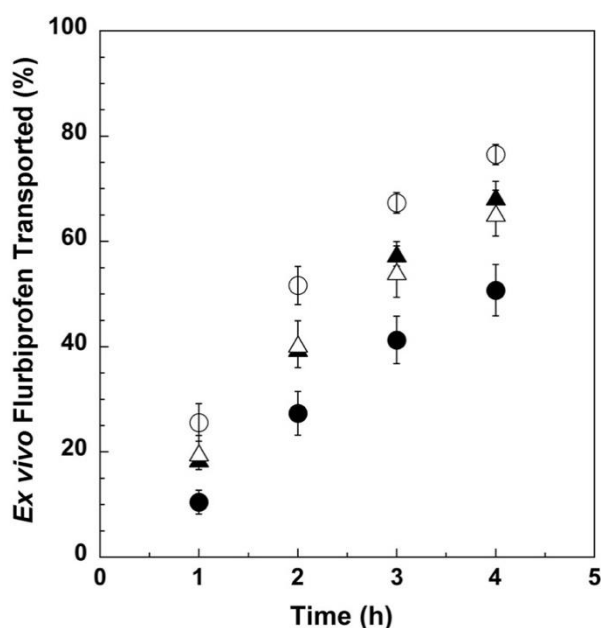
434

435 3.1.2. Soft pellet *in vitro* dissolution and *ex vivo* permeation across nasal mucosa

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

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

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



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

476
 477 In our previous research, the different *ex vivo* permeation of primary microparticles spray-
 478 dried at 70 °C and 40 °C was attributed to the differences in their particle size and
 479 crystallinity (Tiozzo Fasiolo et al., 2019). Blending and agglomeration with the excipient
 480 microparticles eliminated such permeation differences between the two drug microparticle
 481 formulations. However, the soft pellets behaved differently in terms of flurbiprofen
 482 permeation across the mucosa as compared to the corresponding microparticles alone.
 483 After having seen the performance of soft pellets compared to microparticles in the
 484 dissolution tests (Fig. S2 in Supplementary Material), an additional influential action on
 485 flurbiprofen permeation rate from the soft pellet formulation in contact with the slightly wet
 486 mucosa was envisaged.

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

490

491 **3.2 Powder Combination with the Insufflator Device**

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

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

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

514

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

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

524

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

528

529 3.3 Pharmacokinetics in Rat After Nasal Administration

530 Drug absorption into blood across the nasal epithelium occurs by transcellular or
531 paracellular pathways in both the respiratory and olfactory nasal regions (Dhuria et al.,
532 2010). Flurbiprofen is a low molecular weight drug (MW 244.2 g/mol), supporting the
533 transport through the nasal mucosa by both pathways (Lochhead and Thorne, 2012).
534 Following the olfactive or trigeminal nerve routes, a direct transport of drug to brain could
535 also take place along these nervous structures.

536 To study flurbiprofen absorption from the nose and disposition in the brain, the two nasal
537 powders previously selected, namely F13_70 microparticles and soft pellets of F13_40
538 microparticles, were insufflated into the nose of rats. The amount of powder loaded in the
539 nasal device and the corresponding flurbiprofen dose emitted and insufflated into the rat
540 nose are reported in Table II. The powder amount manually metered in the insufflator
541 reservoir ranged between 12-15 mg, complying with the objective to administer similar
542 masses of powder. However, the presence of the excipients used for agglomeration,
543 reduced the dose of flurbiprofen administered with soft pellets, as compared to the same
544 mass of microparticle powder. Consequently, also due to the different amount of powder
545 emitted, the doses of flurbiprofen deposited resulted different. By weighing the insufflator
546 (sensitivity 0.01 mg) before and after the administration, the amount of flurbiprofen made
547 available by the insufflation of microparticles or soft pellets in one nostril was calculated as
548 6.7 mg and 4.2 mg, respectively (see Table II). Finally, for determining the fraction
549 absorbed and brain disposition, intravenous and intranasal solutions of flurbiprofen sodium
550 were administered as well.

551

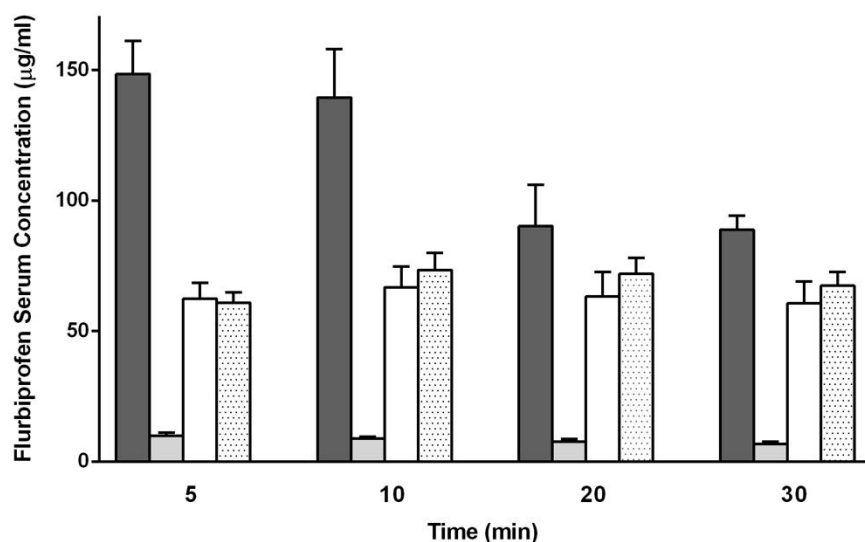


Figure 3. Flurbiprofen serum concentration vs. time after intravenous (IV; dark grey) and intranasal (IN) administrations: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white). Data are expressed as mean \pm SEM ($n \geq 3$).

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 $\mu\text{g/ml}$ and 73.4 ± 6.5 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$ and 67.4 ± 5.2 $\mu\text{g/ml}$, 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

586 the nasal surface for drug absorption was doubled, because two nostrils were engaged
587 during the solution application. However, being limited by flurbiprofen sodium aqueous
588 solubility, the administered flurbiprofen dose was 10 to 20 times as lower as compared to
589 nasal powders. Moreover, the small airway volume of the rat nasal cavity (0.2 cm³; (Xi et
590 al., 2016)) allowed for the administration of maximum 10 µl of drug solution per rat nostril.
591 Accordingly, after flurbiprofen nasal solution administration (0.3 mg drug dose), the
592 obtained serum C_{max} was 10.0 ± 1.1 µg/ml (Fig. 3), that was approximately seven-fold
593 lower than the values measured after nasal powder administration.

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

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

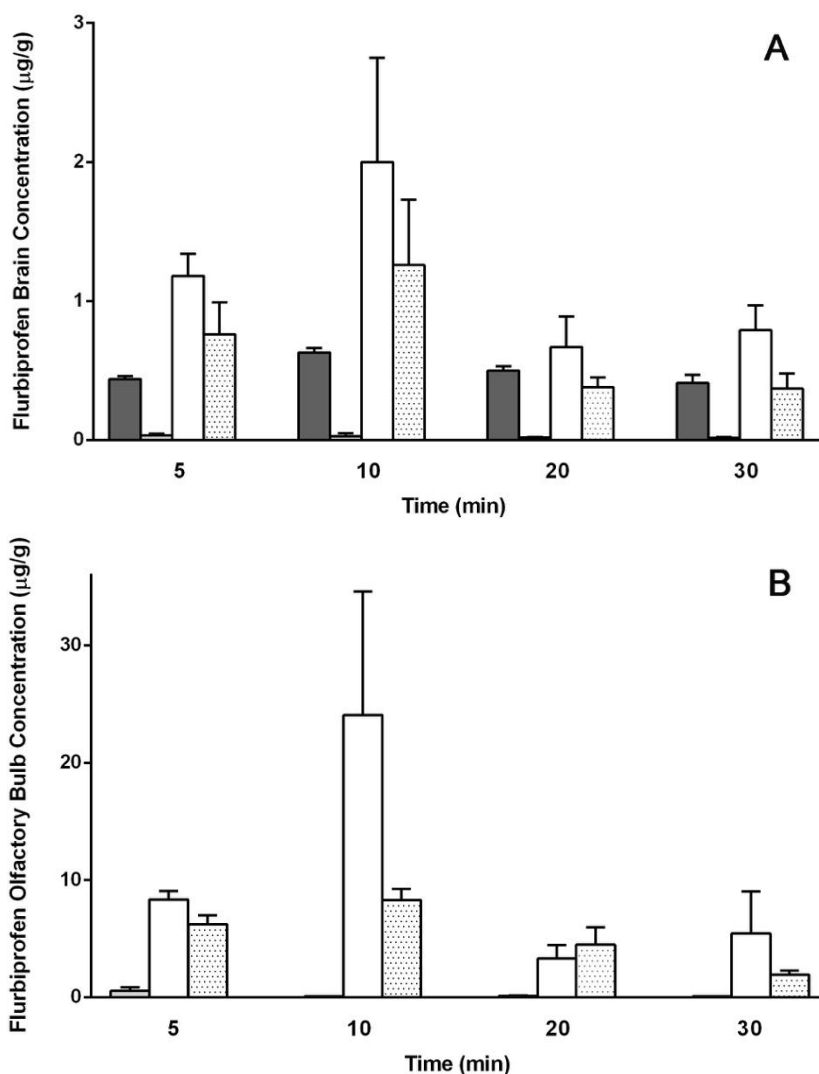
600 In summary, taking as reference the intravenous administration of 4.5 mg of FB-COOH,
601 the nasal insufflation of 6.7 mg of drug as microparticles and of 4.2 mg as soft pellets gave
602 rise to notable fractions of drug absorbed in blood, reaching for the soft pellets the highest
603 absolute bioavailability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

616

617 **3.4 Flurbiprofen Brain Disposition**

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

620 epithelia (Inoue et al., 2020; Lochhead and Thorne, 2012). In this study, both the systemic
 621 and local administration routes enabled an amount of flurbiprofen to access to the central
 622 nervous system (Fig. 4A). After injection of 4.5 mg of flurbiprofen, a maximum level of 0.63 ± 0.03 $\mu\text{g/g}$
 623 ± 0.03 $\mu\text{g/g}$ brain tissue was reached within ten minutes. Interestingly, the highest brain
 624 concentration was measured after intranasal administration of 6.7 mg of FB-COOH as
 625 microparticulate powder ($C_{\text{max}} 2.0 \pm 0.8$ $\mu\text{g/g}$ tissue). Among all administrations, the
 626 superior brain levels obtained with the nasal microparticles were maintained at all time
 627 points. Differently from serum levels, that were quite similar for the two powders, the soft
 628 pellets (4.2 mg of FB-COOH) gave lower brain concentrations than the microparticle
 629 powder ($C_{\text{max}} 1.3 \pm 0.5$ $\mu\text{g/g}$ tissue). Due to the high inter-animal variability, the differences
 630 were not statistically significant ($p > 0.05$).



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

653 vs. intravenous injection (IV; dark grey). Flurbiprofen levels in olfactory bulb were not measured for
654 the IV treatment. Data are expressed as mean \pm SEM ($n \geq 3$).

655

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

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

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

668 As a result of intranasal insufflation of flurbiprofen sodium powders, very significant drug
669 concentrations were measured in the olfactory bulb as compared to rest of the brain (Fig.
670 4B). As this compartment is directly connected to the nasal cavities, drug presence in the
671 bulb signifies direct nose-to-brain transport after intranasal administration.

672 The findings confirm that flurbiprofen administered intranasally as powder form, can
673 directly reach the brain through the nose-to-brain pathway. Moreover, the very high
674 concentration in the olfactory bulb represents a promising aspect of flurbiprofen brain
675 targeting in Alzheimer's disease. In fact, olfactory impairment is recognized as an early
676 sign of AD and other neurodegenerative disorders. It is caused by morphological and
677 signaling alterations of the olfactory nerve (Brai and Alberi, 2018) and correlates with
678 cognitive impairment development. According to Bathini et al. (2019), this suggests that
679 neuronal network imbalances propagate via olfactory bulb and nerve to higher brain
680 centers of the entorhinal cortex and hippocampus. AD initiates in the entorhinal cortex and
681 then spreads outward in an anatomically defined pattern (Adams et al., 2019; Bathini et al.,
682 2019; Holbrook et al., 2020).

683 In summary, the nasal administration of powders enables a significant presence of
684 flurbiprofen in the central nervous system where it is expected to be therapeutic in AD.

685 With these nasal powder dosage forms, it is possible to attain a concentration of
686 flurbiprofen in the brain superior to the one obtained *via* systemic delivery (Lehrer, 2014).

687 After intranasal powder administration, the flurbiprofen accumulation in brain was
688 envisaged to come from the dual contribution to entry through the blood brain barrier (as
689 for IV administration) and through the nose-to-brain direct pathway. Examining the brain
690 disposition, also reported in Table III, flurbiprofen nasal microparticulate powder, in
691 addition to a fraction arrived through the BBB, made available a significant amount of drug
692 directly through the olfactory area in the nasal cavity.
693 Finally, with the nasal solution, FB-COOH presence in the brain at all time points was
694 about 50-fold lower than with the nasal powders. The maximum concentration in the
695 olfactory bulb was $0.55 \pm 0.29 \mu\text{g/g}$ tissue.

696

697 **2.8 Data Analysis by Nose-to-brain Delivery Indexes**

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

706

$$707 \quad DTE = \frac{\left(\frac{AUC_{0-t}(\text{brain})}{AUC_{0-t}(\text{blood})}\right)_{IN}}{\left(\frac{AUC_{0-t}(\text{brain})}{AUC_{0-t}(\text{blood})}\right)_{IV}} * 100 \quad \text{Eq. 2}$$

708

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

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

716

$$717 \quad DTP = \frac{B_{IN} - B_x}{B_{IN}} * 100 \quad \text{Eq. 3}$$

718

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

722

$$723 \quad B_x = \frac{B_{IV}}{P_{IV}} \cdot P_{IN} \quad \text{Eq. 4}$$

724

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

727 According to Kozlovskaya et al. (2014), the value of DTP can range from $-\infty$ to 100.

728 However, we believe that values equal to zero or negative indicate that the drug is
 729 delivered to the brain essentially *via* BBB.

730 The interest of these values is that they are independent of the different doses
 731 administered. In this study, DTE values of the nasal powders were notably higher than
 732 100, identifying a more efficient nasal brain targeting compared to IV injection. In contrast,
 733 the nasal solution was less efficient, exhibiting a value lower than 100 (Table III). In detail,
 734 following intranasal powder administration, the brain targeting efficiency was more
 735 consistent with the nasal microparticles than with the soft pellets, i.e., DTE 456% vs 251%,
 736 respectively.

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

745

746 *Table III. Area Under the Curve (AUC) calculated in serum and in brain, Drug Targeting Efficiency*
 747 *(DTE) and Direct Transport Percentage (DTP) of flurbiprofen for intranasal drug powders, namely*
 748 *F13_70 microparticles and F13_40 soft pellets, and drug solution compared to IV administration.*

749 *AUC data are expressed as mean \pm SEM.*

Treatment	AUC_{0-t} serum ($\mu\text{g ml}^{-1} \text{ min}$)	AUC_{0-t} brain ($\mu\text{g g}^{-1} \text{ min}$)	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

750

751 The microparticulate powder enabled a higher direct traffic of flurbiprofen from the nasal
752 cavity to the brain than soft pellets. The nose-to-brain direct transport relies on deposition
753 and retention of the powder at olfactory region. With soft pellets, in which the drug is
754 diluted by excipients, the drug amount deposited per unit epithelium area is expected to be
755 less favorable. The high bioavailability obtained with the soft pellets powder suggests an
756 important deposition in the respiratory region. Better coverage of the olfactory mucosa by
757 the microparticulate pure drug powder may have favored nose-to-brain transport. It is
758 known that the shape of the plume emitted from a device and the deposition of particles
759 within nasal cavities are influenced by the properties of powder formulation (Buttini et al.,
760 2012). This leads to different particle lining of the mucosal surface, either respiratory or
761 olfactory. Soft pellets have lower aerosolization performance in terms of de-agglomerated
762 particle size emitted by nasal device, compared to microparticles. In fact, in a study
763 regarding the technological development of soft pellets of caffeine spray-dried
764 microparticles for nasal delivery, Russo and co-workers (2004) reported that during
765 insufflation the agglomerates were broken in fragments with significantly reduced size. Still
766 these fragments were larger than the original microparticles. More specifically, the size of
767 fragments was dependent on the agglomerate's mechanical resistance (Adi et al., 2011).
768 This size difference ultimately affects the site of drug dissolution and transepithelial
769 transport (Buttini et al., 2012; Tiozzo Fasiolo et al., 2018).

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

780

781 **CONCLUSIONS**

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

785 Compared to intravenous administration, flurbiprofen sodium powders, insufflated into the
786 nose, enhanced the drug concentration in brain, despite the lower drug serum
787 concentration. The Direct Transport Percentage index evidenced that at least 60% of the
788 intranasal dose reached the brain via direct nose-to-brain transport for both powders.

789 Nasal soft pellets, very effective in dose delivery, showed a fraction of drug absorbed
790 through the respiratory epithelium, higher than the primary microparticles. However, nasal
791 microparticle powder outperformed the soft pellet powder in the direct transport of
792 flurbiprofen to brain. The very high drug concentration in the olfactory bulb measured for
793 microparticulate powders, substantiates the direct nose-to-brain drug transport. The
794 deposition of microparticles by nasal insufflation into rat nasal cavity resulted in larger
795 surface of olfactory mucosa covered by impacted particles, hence, sustaining the drug
796 passage to brain along olfactory epithelium.

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

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

809

810

811 **ACKNOWLEDGMENTS**

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813 System device for the *in vivo* experiments.

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

817

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820 commercial, or not-for-profit sectors.

821

822 **COMPETING INTEREST STATEMENT**

823 The authors declare no competing interests.

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978 **SUPPLEMENTARY MATERIAL**

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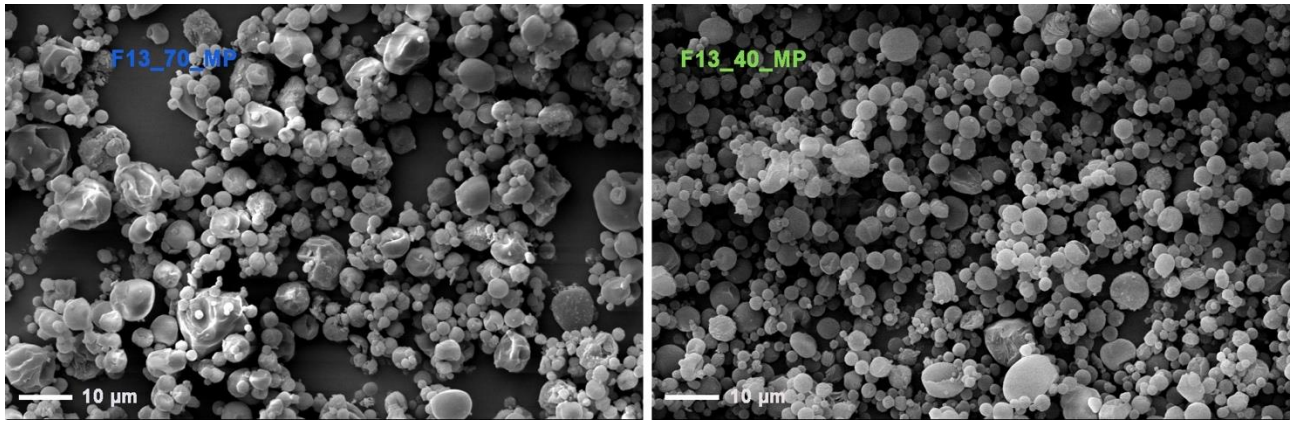
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988 Figure S1. SEM micrographs (2500x; size bar: 10 µm). From left to right: FB-COONa
989 microparticles spray-dried at 70 °C (F13_70_MP); FB-COONa microparticles spray-dried at
990 40 °C (F13_40_MP).

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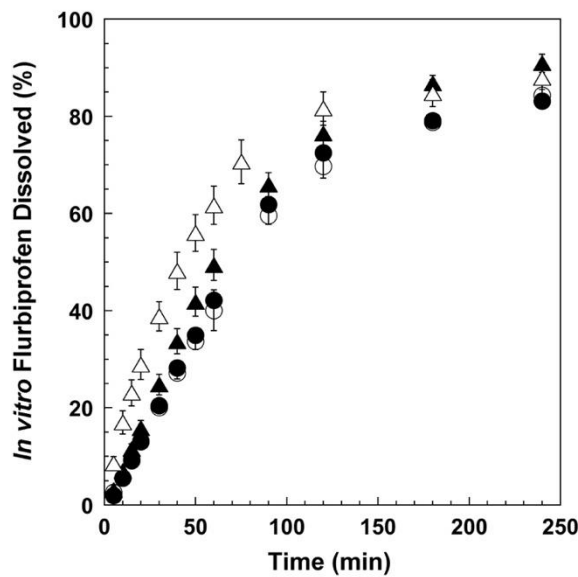
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1005 Figure S2. Flurbiprofen dissolution/transport across a regenerated cellulose membrane
1006 from soft pellets (triangle) of F13_40_MP (white) and F13_70_MP (black) vs. the primary
1007 microparticles (circle) F13_40_MP (white) and F13_70_MP (black) (mean ± SEM, n=3).
1008 Data for the microparticles have been re-elaborated from Tiozzo Fasiolo L. et al. Tiozzo
1009 Fasiolo et al., 2019).

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International Journal of Pharmaceutics

Ferrara, March 12th, 2021

Object: **Research Article submission**

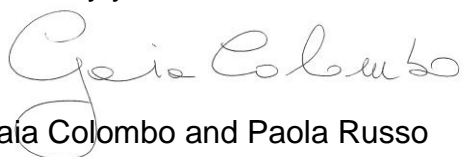
Dear professors,

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.

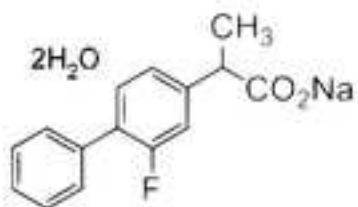
Thank you.

Sincerely yours,

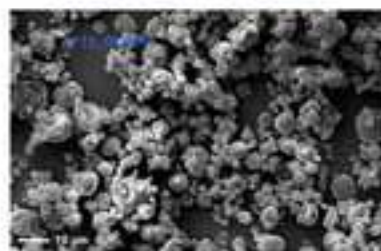


Gaia Colombo and Paola Russo

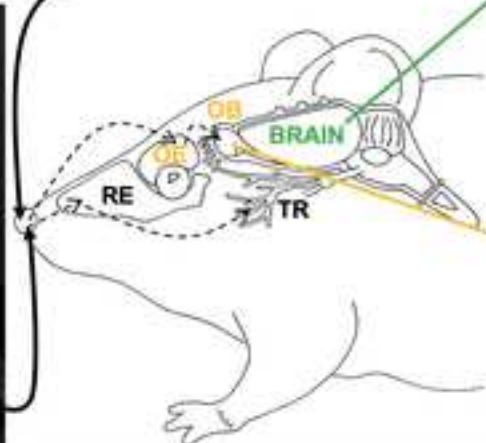
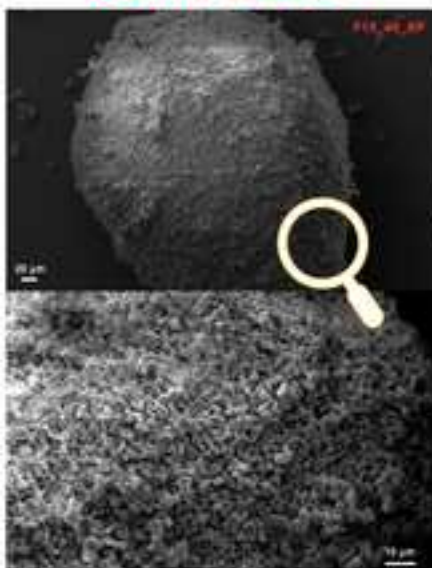
FLURBIPROFEN SODIUM NASAL POWDERS IN EARLY ALZHEIMER'S DISEASE



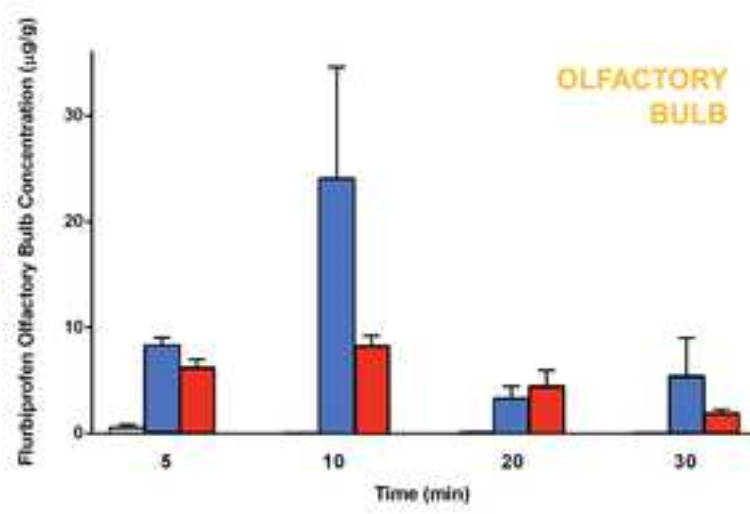
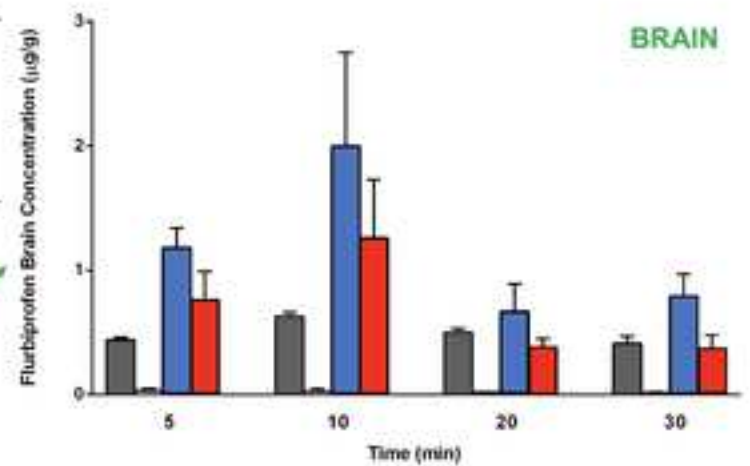
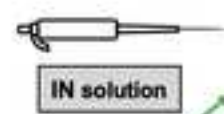
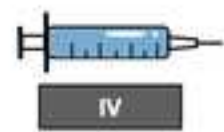
SPRAY-DRIED MICROPARTICLES



SOFT PELLETS



RE: respiratory epithelium
 OE/OB: olfactory epithelium/bulb
 TR: trigeminal nerve



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 \pm 2.3
F13_40_MP	50:50	35.0 \pm 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 \pm standard deviation ($n \geq 13$).

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

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

Figure 1

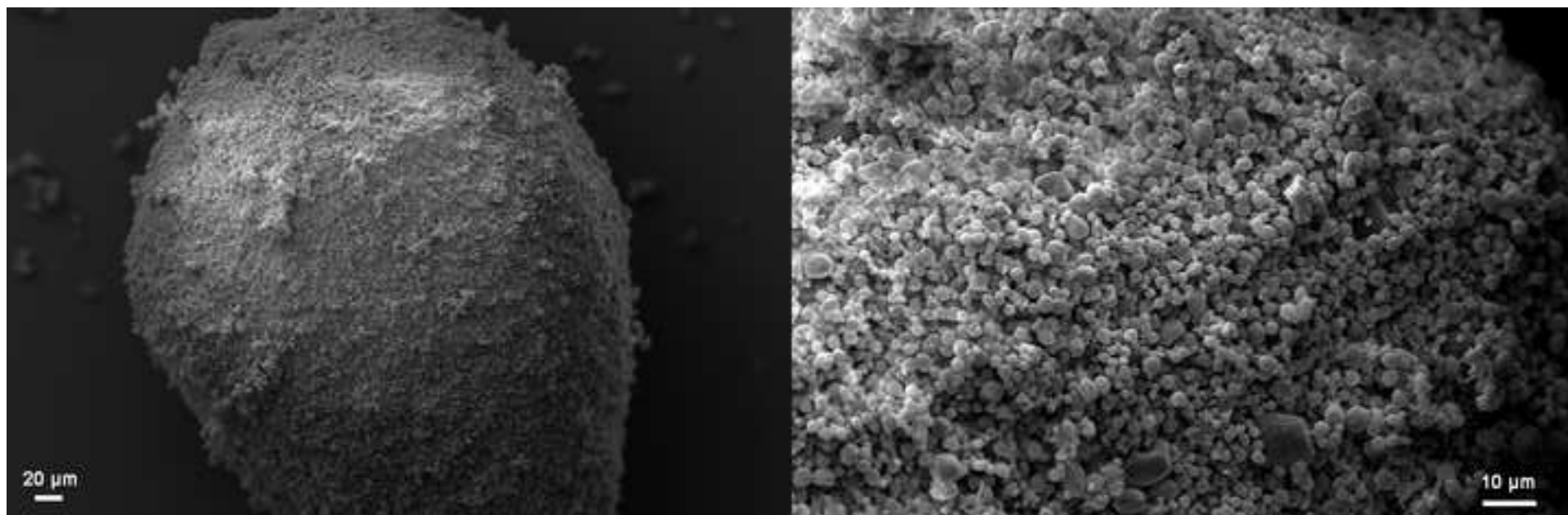


Figure 2

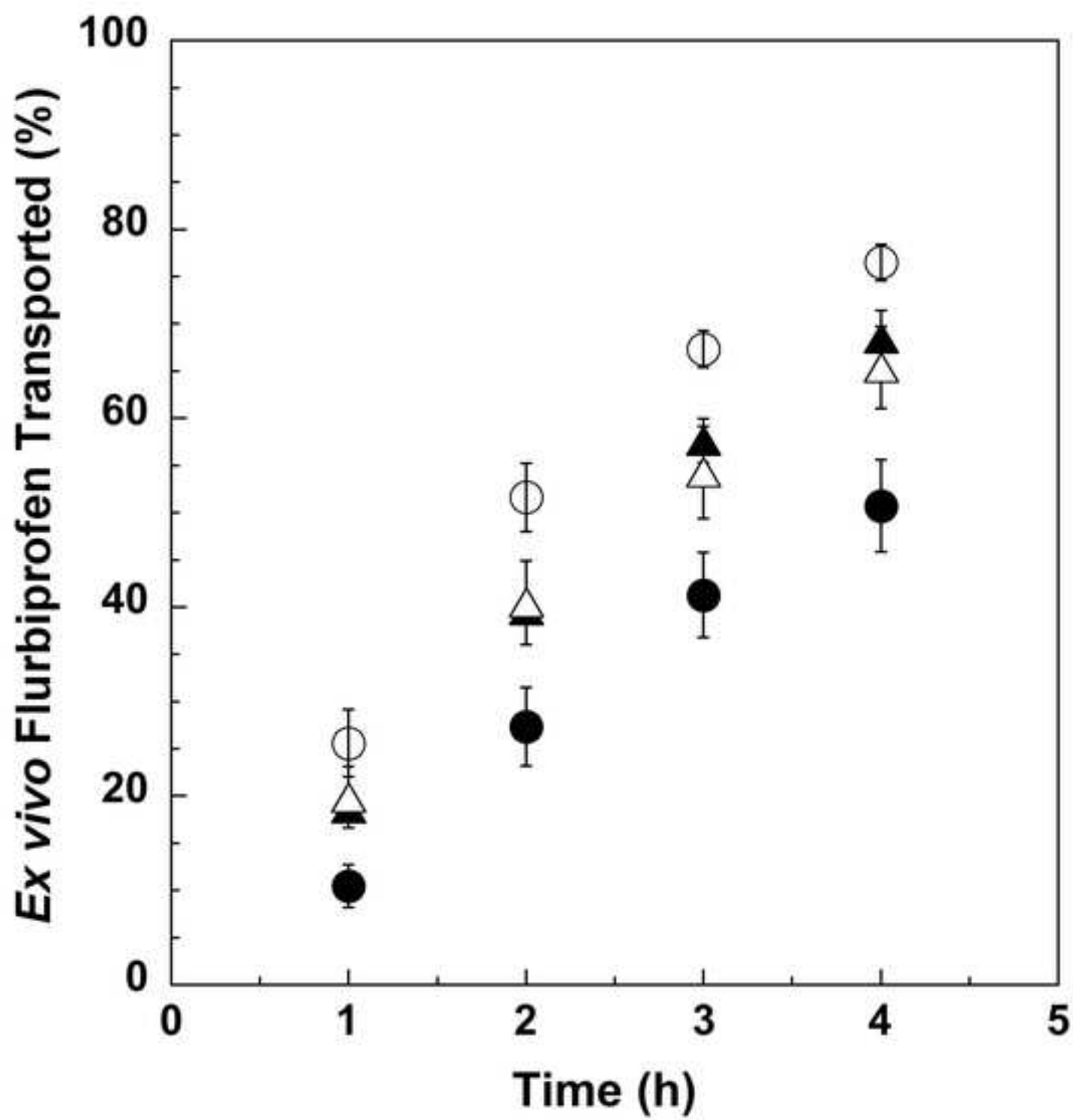


Figure 3

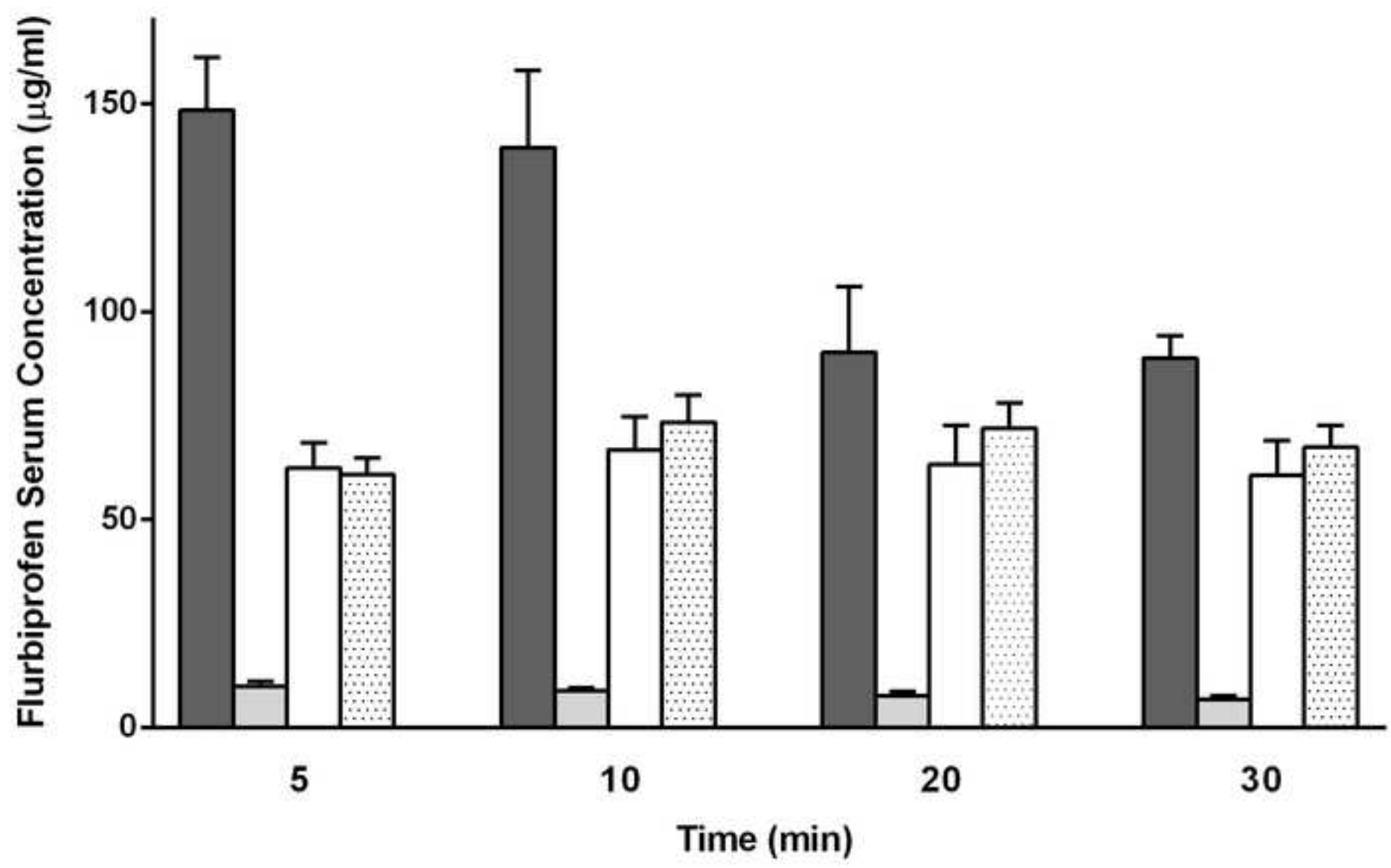


Figure 4, panel A

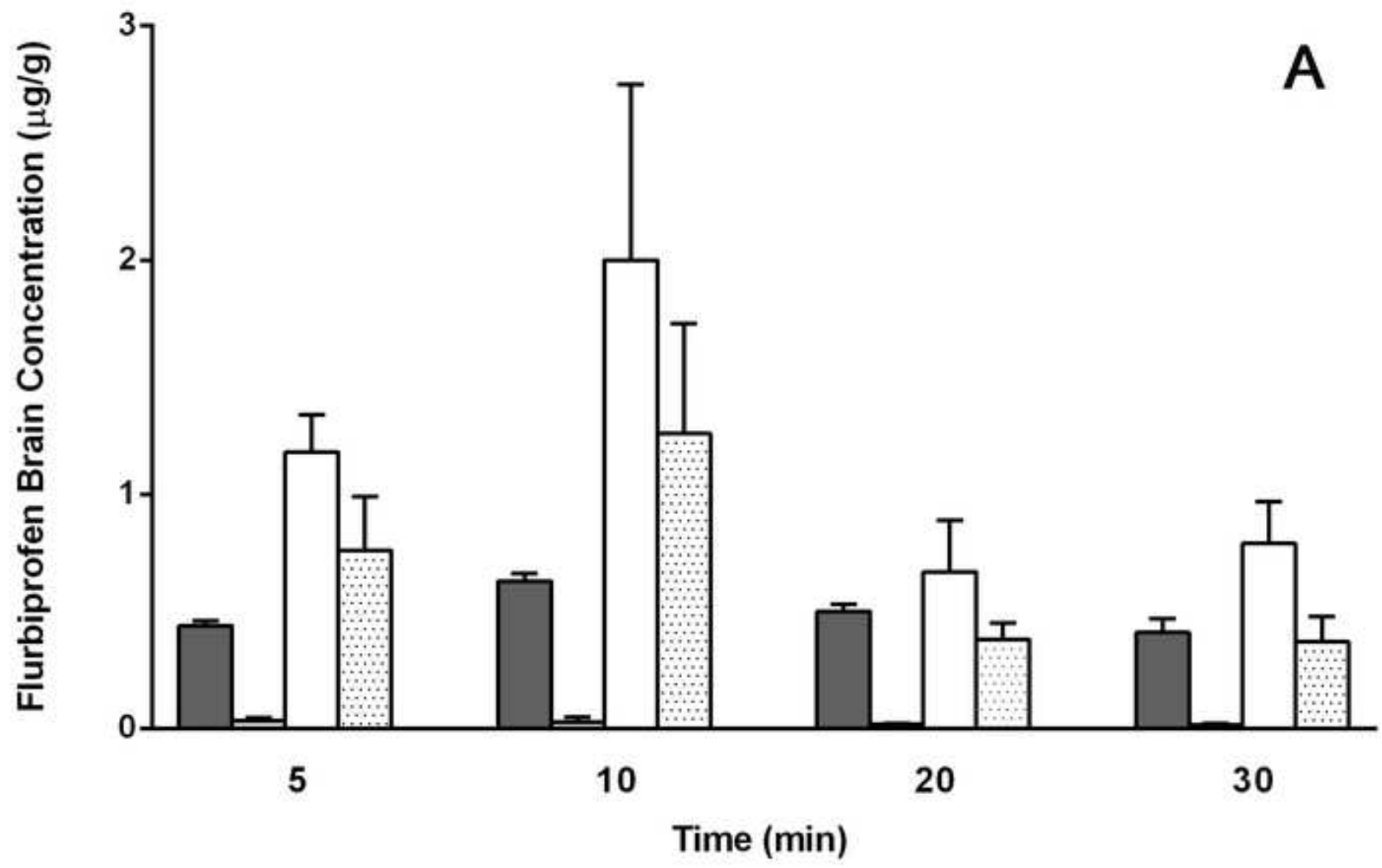


Figure 4, panel B

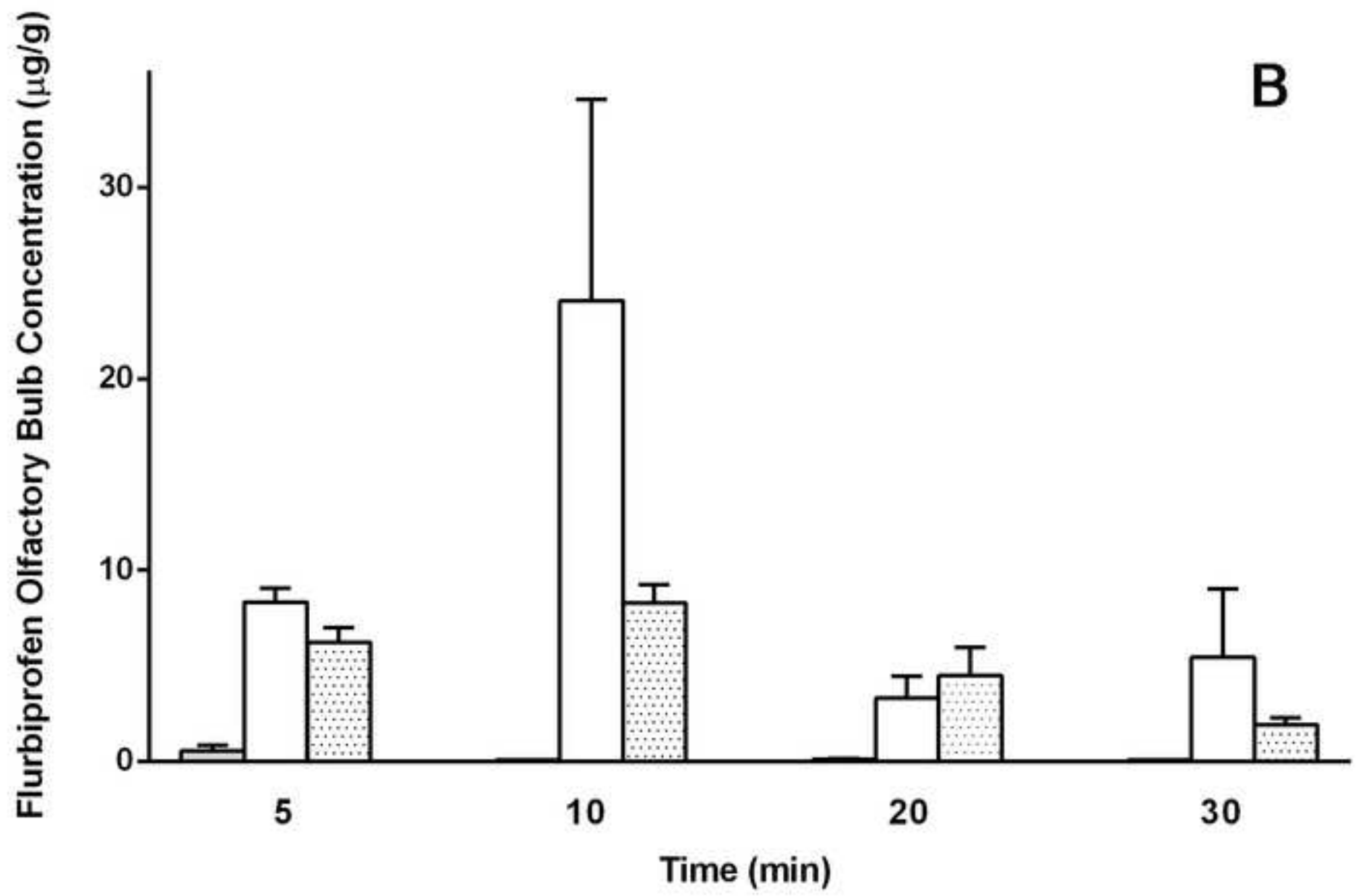



FIGURE CAPTIONS

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 \pm SEM, $n \geq 5$). 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 \pm SEM ($n \geq 3$).

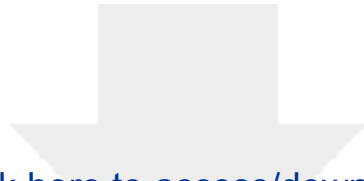
Figure 4. Flurbiprofen concentration in brain (panel A) and olfactory bulb (panel B) after intranasal (IN) administration of: IN solution (light grey), IN microparticles (white), IN soft pellets (dotted white) vs. intravenous injection (IV; dark grey). Flurbiprofen levels in olfactory bulb were not measured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



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