1 **Comprehensive two- dimensional liquid chromatography as a biomimetic screening platform for**

2 **pharmacokinetic profiling of compound libraries in early drug development.**

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Comprehensive two- dimensional liquid chromatography as a biomimetic screening platform for pharmacokinetic profiling of compound libraries in early drug development.

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Abstract

A comprehensive two-dimensional liquid chromatography-based biomimetic platform (LCxLC) has been developed and validated for drug diffusion studies. Human serum albumin and Immobilized Artificial Membrane were thereby used in the first (^{1}D) and second (^{2}D) separation dimension, respectively. While the former was meant to emulate the blood, the latter was instead intended to mimic the intestinal mucosa epithelium. Therefore, the experimental conditions, *i.e.* pH, temperature and buffer composition, were modulated to reflect faithfully *in vivo* conditions. 30 compounds, whose effective intestinal permeability (P_{eff}) assayed *in situ* on humans by a validated technique was known from the literature, were used as model drugs.

48   A good and orthogonal separation was achieved for the whole dataset, although for a better

49   distribution of the most polar compounds in the elution window a segmented gradient elution

50   program had to be employed. Interestingly, the passively uptaken compounds having the most

51   favorable P_{eff}, populated a specific area of the 2D plots, implying that the affinity for HSA and IAM

52   has to lie in specific ranges in order for a compound to be satisfactorily absorbed from the

53   intestinal lumen.

54   Although these results should be regarded as preliminary, this work paves an entirely new and

55   unprecedented way to profile pharmaceutically relevant compounds for their *in vivo* absorption

56   and distribution potential.

57

58   *Keywords:* Immobilized Artificial Membrane; Human Serum Albumin; effective intestinal

59   permeability; comprehensive two-dimensional liquid chromatography; ADMET profiling;

60   bioaffinity chromatography.

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Graphical abstract

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1. Introduction

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Most of the drug formulations are nowadays available on the market as oral dosage forms, being

the oral administration route safer, cheaper and capable of meeting superior patient compliance

[1]. Consequently, the majority of the active pharmaceutical ingredients (APIs) have to pass the

intestinal barrier to obtain the pharmacological effect for which they were designed [2]. For this

reason, intestinal absorption represents a key biopharmaceutical feature to assess the

performance of a drug with direct effects in drugs' bioavailability (BA) [3]. Since 1995 the

Biopharmaceutic Classification System (BCS) classifies the drugs into four classes according to their

dose related-solubility and intestinal permeability [4].

84   In brief, some drug products could be considered for biowaiver, *i.e.* approved by providing only

85   dissolution test outcomes rather than envisaging full bioequivalence studies on human subjects,

86   with the proviso that (*a*) these formulations are immediate-release (85% of the drug released

87   within 30 minutes) (*b*) the dose is fully soluble in water over a 1.0-7.5 pH range (*c*) the intestinal

88   absorption is almost quantitative (fraction absorbed >90%) [5]. While water solubility is fairly easy

89   to measure, permeability should be instead properly assayed to achieve correct BCS classification

90   [6]. For instance, the food and drug administration (FDA) currently accepts absorption data only if

91   intestinal uptake evidence is produced by human pharmacokinetic (PK) studies (mass balance or

92   absolute BA) or *in vivo* intestinal perfusion in human subjects [7]. Alternatively, *in vivo* or *in situ*

93   intestinal perfusion methods based on murine models, as well as models based on excised tissues

94   or cellular monolayers, can as well be used. However, these procedures must be properly

95   validated and even in that case their reliability is still regarded with extreme caution, as

96   interlaboratory variability of data based on cell protocols is generally high. Indeed, uptake studies

97   performed on such systems are claimed trustworthy only if the absorption is demonstrated to

98   occur exclusively by passive diffusion, *i.e.* without any involvement of ATP-operated protein

99   channels [7]. Consistently, the FDA encourages biowaiver applications providing intestinal

100   absorption outcomes from different methodologies and in case of conflicting information, human

101   data supersede *in vitro* or animal data.

102   The gold standard of permeability determinations has long been considered the intubation and

103   perfusion of a drug solution *in situ* in healthy human volunteers [2]. This approach (so called LOC-I-

104   GUT perfusion technique) has been used for over 50 years, and effective permeability (P_{eff}) values

105   determined by this method give the best indication as to whether a drug compound has a

106   sufficient potential for absorption. In brief, to allow the perfusion of a drug solution in a human, a

107   radiopaque multichannel perfusion tube is inserted through the mouth and positioned in a region

108   comprised between the proximal to mid-small intestine [8]. Once the tube is correctly placed, a

109   perfusion solution is continuously administered through one of the channels in the tube, and

110   subsequently collected from other channels. P_{eff} is determined by measuring the disappearance of

111   an API from the intestinal lumen by perfusion and is equal to:

112    $$P_{eff} = \frac{-Q_{in}*\ln\left(\frac{C_{out}}{C_{in}}\right)}{A}$$ Eq. (1)

113   where Q_{in} is the perfusate flow rate, C_{out} and C_{in} are the concentrations of API leaving and entering

114   the intestinal segment, and A is the surface area of the perfused intestinal segment, which is

115   assumed to be a smooth cylinder [8]. However, as human intestinal perfusion requires patients'

116   hospitalization and follow-up, this technique is time- and resource- consuming. Due to these

117   considerations, albeit valuable, human intestinal perfusion work is hardly performed.

118   Alternative methods, based on separation science, are increasingly gathering the attention of the

119   scientific community [9, 10]. These methods allow the introduction in the separation mode of

120   biological structures playing an essential role in drugs' absorption such as membrane

121   phospholipids and plasma proteins [11, 12]. The advantage lies in (*a*) a superior reproducibility of

122   the measurements as compared to data achieved by the culturing of suitable cell monolayers, *e.g.*

123   Caco-2 cells, (*b*) their greater speed as compared to screening methodologies implemented on

124   animal (human included) models and (*c*) the aspect that, being based on physico-chemical

125   parameters, they allow elucidation of molecular mechanisms.

126   Chiral protein-based stationary phases, such as those based on human serum albumin (HSA), are

127   commercially available and were successfully used for both chiral separation and plasma protein

128   binding assessment purposes in high performance liquid chromatography (HPLC) set-ups [13]. HSA

129   is indeed the most abundant plasma protein found in human blood. Produced by the liver, HSA

130   has a 35-50 g L^{-1} reference blood concentration range and binds preferably acidic compounds [14].

131   Although HSA typically engages with analytes through non-specific hydrophobicity-driven

132 interactions, certain protein domains were demonstrated to be capable to recognize enantiomers

133 thanks to the presence of specific interaction sites. At least two major enantioselective sites, *i.e.*

134 Site I (binding warfarin) and Site II (binding diazepam), have been described on HSA [15].

135 Immobilized artificial membrane (IAM) are stationary phases based on phosphatidylcholine (PC)-

136 analogues covalently bound to a silica core exploited in reversed phase HPLC [16, 17]. They

137 represent a rather simplified model of complex lipoidal biological bilayers with the main

138 shortcomings being the monolayered nature of the membrane and the aspect that only one

139 phospholipid, *i.e.* PC, is represented. Nevertheless, IAM-HPLC was proved effective in predicting

140 the extent at which drugs cross biological membranes of strategic relevance in biopharmaceutics

141 such as the blood-brain barrier, the intestinal tract and the skin [18-25].

142 To the best of our knowledge, to date either IAM or HSA HPLC have been extensively and

143 successfully employed in only one-dimensional liquid chromatography approaches for the

144 characterization of pharmacokinetic and biodistribution properties of APIs. In recent years, the

145 need of separating and characterizing more and more complex samples has led to the

146 development of increasingly smaller particles and consequently of hardware capable of

147 withstanding comparatively higher operating pressure [26]. However, this trend seems to have

148 terminated in its natural evolution and chromatographers and separation scientists have focused

149 their attention on multidimensional liquid chromatographic approaches as more powerful

150 solutions to generate superior separation capacity [27].

151 In this work, a comprehensive two-dimensional platform has been for the first time developed in

152 fully biomimetic separation conditions and applied on 30 compounds whose intestinal effective

153 permeability was known from one single bibliographic source to exclude any interlaboratory

154 variability. The achieved separation has been eventually evaluated from both an analytical and a

155 biopharmaceutical perspective.

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157

2. Materials and Methods

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2.1 Chemicals and reagents

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The solutes were obtained from Merck (Machelen, Belgium), TCI-Europe (Zwijndrecht, Belgium), Thermofisher Acros Organics (Geel, Belgium) and Sanbio (Uden, The Netherlands) as listed in Table 1. Naproxen, (+)-warfarin and (-) warfarin were purchased from Merck. The purity of all the tested solutes was equal to or higher than 98%. Water (18.2 MΩ·cm^{-1}) was purified and deionized in house via a Milli-Q plus instrument from Millipore (Bedford, New Hampshire, USA). Acetonitrile and methanol used for the preparation of the eluents were HPLC grade and obtained from Biosolve (Valkenswaard, The Netherlands). Potassium phosphate monobasic and ammonium acetate were both from Sigma-Aldrich (Machelen, Belgium) and their purity was equal to and higher than 99%.

171

2.2 LC analytical columns

173

The ^{1}D column was a Chiralpak HSA 150 x 2 mm i.d., 5 μm (Daicel, Raunheim, Germany) used with a Chiralpak 10 x 4 mm guard cartridge inserted in a cartridge holder and a column coupler (both for protein-based analytical columns), while the ^{2}D column was a IAM.PC.DD2, 150 x 4.6 mm i.d., 10 μm (Regis Technologies, Morton Grove, USA).

178

2.3 2D LC system

180

181 The ^{2}D LC instrument was assembled from two Agilent 1100 systems (Agilent Technologies,

182 Waldbronn, Germany), interfaced via a 2-position/10-port switching valve with a microelectric

183 actuator (VICI, Houston, U.S.A., model C2H-2000EH). The ^{1}D separation was performed using an

184 1100 quaternary pump equipped with a 1100 degasser coupled to an external six-port injection

185 valve (Rheodyne, Alsbach, Germany). The temperature was controlled by an Agilent 1100 Series

186 Thermostatted Column Compartment. An 1100 variable wavelength detector (VWD) equipped

187 with a micro flow cell was used to monitor the ^{1}D separation. The ^{2}D instrument consisted of an

188 1100 binary pump, 1100 degasser, and 1100 VWD equipped with a standard flow cell. All modules

189 were controlled using two Windows-based computers equipped with ChemStation software

190 (Agilent). The first was used to control the ^{1}D pump, ^{1}D detector, and ^{2}D detector, and the second

191 computer was used to operate the ^{2}D gradient on the ^{2}D pump. The 10- port switching valve was

192 equipped with two 100 µL loops, and the modulation time was 2.0 min.

193

194 **2.4. Competitive binding and HSA Site I occupancy assessment**

195

196 *2.4.1 Mobile phases*

197

198 The HSA mobile phase was composed of a 100 mM ammonium acetate buffer solution (A) and a

199 75/25 (v/v) ammonium acetate buffer 100 mM pH 7/acetonitrile (B). The pH was adjusted at pH

200 7.0 by dropwise addition of ammonia. The mobile phase gradient applied on the IAM column in

201 the ^{2}D comprised a 10 mM potassium phosphate monobasic (Sigma-Aldrich, Machelen, Belgium

202 purity $\geq$ 99%) buffer solution (A) and a 45/55 (v/v) 10 mM potassium dihydrogen

203 phosphate/acetonitrile (B) solution. The pH of this buffer was adjusted at pH 5.0 *via* dropwise

204 addition of hydrochloric acid.

205

206 *2.4.2 2D LC elution programs*

207

208 The ^{1}D separation was carried out at a 50 μL min^{-1} flow rate and under controlled temperature (30

209 °C) by using a linear gradient elution program set as follows: 0 min: 0% B$_1$; 10 min: 0% B$_1$; 20 min:

210 40% B$_1$; 40 min: 50% B$_1$; 60 min:50% B$_1$; 100 min: 85% B$_1$; 180 min: 100% B$_1$; 200 min: 100% B$_1$.

211 The ^{2}D separation was carried out at 5.0 mL min^{-1} and at room temperature, *i.e.* 25 ± 2 °C, by using

212 a linear gradient elution program set as follows: 0 min: 25% B$_2$; 1.50 min: 100% B$_2$; 1.51 min: 25%

213 B$_2$; 2 min: 25% B$_2$.

214

215 **2.5 Bioaffinity measurements**

216

217 *2.5.1 Mobile phases*

218

219 The HSA mobile phases consisted of a 100 mM potassium phosphate monobasic buffer solution

220 (A) and a 75/25 (v/v) a 100 mM potassium dihydrogen phosphate/2-propanol (HPLC grade,

221 Biosolve, Valkenswaard, The Netherlands) solution (B). To mimic the blood compartment, the pH

222 was adjusted with hydrochloric acid and the aqueous solution had a pH value of 7.00 ± 0.05. The

223 IAM mobile phases were the same as that used for the calibration and described in 2.4.1.

224

225 *2.5.2 2D LC elution programs*

226

227   Two different separation methods were developed. In both cases, the ^{1}D separation was carried

228   out at a 50 µL min^{-1} flow rate and under controlled temperature (30 °C) by using a linear gradient

229   elution program set as follows: 0 min: 0% B_1; 20 min: 0% B_1; 120 min: 100% B_1; 200 min: 100% B_1.

230   In both analytical methods, the ^{2}D separation was carried out at 5.0 mL min^{-1} and at room

231   temperature, *i.e.* 25 ± 2 °C. However, in a first method a fixed linear gradient ^{2}D elution program

232   was set as follows: 0 min: 25% B_2; 1.50 min: 100% B_2; 1.51 min: 25% B_2; 2 min: 25% B_2, whereas in

233   a second method a mixed gradient elution method was used. Therefore, from 0 to 36 min the

234   program was set as follows: 0 min: 0% B_2; 1.50 min: 55% B_2; 1.51 min: 0% B_2; 2 min: 0% B_2 and

235   from 36 to 200 min the composition of the eluents was changed as follows: 0 min: 25% B_2; 1.50

236   min: 100% B_2; 1.51 min: 25% B_2; 2 min: 25% B_2.

237   After preparation, all the mobile phases were *vacuum*- filtered through 0.20 µm nylon membranes

238   (Grace, Lokeren, Belgium) and degassed in an ultrasonic cleaner (Branson 2510, Frequency: 40

239   kHz, Branson Ultrasonics, Danbury, USA) for 20 minutes before use.

240

241   **2.6 Sample preparation**

242

243   Stock solutions of all drugs were prepared by dissolving 10 mg of each solute in 2 mL of methanol

244   and kept at -4 °C, except for cyclosporin A which was dissolved in DMSO and stored -20°C.

245   Working solutions were freshly prepared at the beginning of each day by dilution of the stock

246   solutions to 50 µg mL^{-1} with the starting mobile phase for all the analytes. Nifedipine samples and

247   nifedipine-containing mixtures were wrapped in aluminium foil before feeding the autosampler to

248   protect this chemical from photodegradation.

249

250   **2.7 LC experimental conditions**

251

252 The injection volume for each analysis was 10 μL. The calibration was recorded at 254 nm UV

253 wavelength whereas the bioaffinity measurements were monitored at 220 nm UV wavelength.

254

255 **2.8 Postprocessing**

256

257 Raw data were exported as comma-separated values and converted to a data matrix in GC image

258 R2.5 software (GCimage, Lincoln, U.S.A.). From these matrices, contour plots and 3D scatterplots

259 were generated by using OriginPro 8.5 (OriginLab Corporation, Northampton, U.S.A.). The

260 chromatographic retention factors of each analyte were calculated by using Eq. (S1).

261

262 **2.9 Data sources**

263

264 For the scatterplots, n-octanol/water lipophilicity values either of the neutral forms of the

265 analytes, $i.e.$ log P^N, and of the mixtures present in solution at pH 7.4, $i.e.$ log $D^{7.4}$ were collected

266 from the scientific literature [28, 29] (except for creatinine, fexofenadine, whose log P^N and log

267 $D^{7.4}$ values were calculated and for cyclosporin A, lisinopril, losartan, L-phenylalanine and

268 valacyclovir, whose log $D^{7.4}$ but not log P^N values were calculated), whilst all n-octanol/water the

269 mixtures present in solution at pH 5.0, $i.e.$ log $D^{5.0}$, were calculated, except for acetaminophen, (+)-

270 griseofulvin and nifedipine, which do not support any ionizable moiety and, as a consequence of

271 that, their log P^N values were assumed as reasonable estimates of their log D values at both pH

272 values. The calculation of lipophilicity values was accomplished by the software Marvin Sketch

273 17.1.23.0 on a Windows-based quad-core PC [30]. Effective human jejunum permeability (P_{eff})

274   data were taken from [31, 32]. When more than one P_{eff} value was reported, an average was

275   considered.

276

277

278   **3.0 Results and Discussions**

279

280   **3.1 Method development**

281

282   Protein-based liquid chromatographic stationary phases are generally used for bioaffinity

283   chromatography. Therefore, method development conducted on such phases, even in 1D, poses

284   several constraints, regardless of these being exploited for protein binding assessment or for mere

285   chiral separation purposes. Indeed, deliberate deviations from the recommended pH range (5.0-

286   7.0), temperature (20-30 °C), operating pressure (lower than 150 bar), buffer concentration (up to

287   100 mM) and types (ammonium acetate or phosphates), organic modifier ratio (0-15% v/v for

288   analysis, max 25% for decontamination) and type (2-propanol or in general alcohols preferred) can

289   lead to significant reduction of column lifetime and loss of reproducibility [33]. The manufacturer

290   suggests the usage of charged additives such as N,N-dimethyloctyl amine (DMOA), trifluoroacetic

291   acid (TFA), octanoic acid (OA), heptafluorobutyric acid to be added to the mobile phases at a

292   concentration not higher than 2, 10, and 5 mM, respectively to modulate retention and

293   enantioselectivity [33].

294   However, in our study this was not an option since, as the manufacturer acknowledges, these

295   additives have such a strong affinity for the matrix that they end up being very difficult, if not

296   impossible, to remove quantitatively. Consistently, the manufacturer suggests that if the

297 separation scientist wishes to include such cationic or anionic additives as part of method

298 development then "the column should be dedicated for the purpose".

299 This work is aimed at prospecting the selectivity of two different stationary phases embedding

300 biological structures that are crucial for drug absorption (IAM) and distribution (HSA) in a 2D LC

301 setting. For this reason, in the method development we were concerned not only on allowing a

302 satisfactorily separation of the analytes under consideration, but also the on ensuring that the

303 analyses were realized in conditions able to mirror as closely as possible the biological

304 compartment in which absorption and distribution take place. To fulfil this goal, we worked to

305 assure that the affinity indexes achieved from the biomimetic measurements had solid physico-

306 chemical meaning and accuracy and that these both were retained over time.

307 Consistently, the utmost care was put in preserving the performance of the biomimetic LC

308 columns over time. This was achieved by optimizing on one hand the capability of the separation

309 process to account for the highly specific recognition forces and molecular interactions that occur

310 *in vivo.* On the other hand, we compromised for a longer column lifetime and no deviation from

311 the ideal chromatographic behaviour that these phases feature. For instance, although some

312 authors [9] conducted plasma protein binding measurements on HSA column applying conditions

313 differing from those recommended by the manufacturer (high flow rates, *i.e.* 1.8 mL min^{-1} on a 50

314 x 3 mm, mobile phase at pH 7.4, 30% (v/v) 2-propanol), we preferred to strictly adhere to these,

315 even if this may effect in longer run times. This is because we designed this biomimetic platform

316 with a potential to be exploited in drug development programs in which large compound libraries

317 are assayed daily and data reproducibility is crucial. Consistently, although the pH of the blood

318 compartment in physiological condition is 7.4, the ^{1}D mobile phase pH was set to 7. This is

319 motivated by the instance that the HSA column should not be used at a pH above 7.0 according to

320 the column manufacturer [33]. A pH 5 was chosen to mimic that of the duodenum and the small

321 intestine. These are the regions of the gastrointestinal tract that are mainly involved in drugs'

322 absorption, thanks to the very large surface area (*ca.* 80 cm^2 cm^{-1}) that they provide along with

323 their typically long transit time (>3 h) [34].

324

325 The results of a performance check are described in section S1.0 of supporting information

326 whereby representative chromatograms are shown in Figure S1 and S2 along with the

327 experimentally determined chromatographic retention coefficients and resolution values which

328 are listed in Table S1. The HSA column features enhanced affinity for neutral and especially acidic

329 compounds and the capability of the stationary phase to resolve a (±)-warfarin racemate was

330 assumed as an indication of Site I being intact. This assures that the HSA affinity indexes depict not

331 only unspecific – generally lipophilicity- driven – interactions, but also highly specific recognition

332 forces, which are responsible of enantioselectivity. On the contrary, the IAM phase exhibits

333 superior retention of cations, whereas acids are on average less retained than neutral compound

334 having same lipophilicity values in agreement with Avdeef's "pH piston hypothesis" [35].

335 According to this, cations would be favored with regards to neutral compounds of same

336 lipophilicity in the interaction with IAM.PC phases as its negatively charged phosphate moieties

337 locate more internally as compared to the positively charged amino groups. This allows bases to

338 have a deeper and more productive interaction of electrostatic nature and to better

339 accommodate their apolar moieties in the hydrophobic tails of the lipid network, especially as

340 compared to acidic solutes. Therefore, in our design we chose to conduct the separation in ^{1}D on

341 the HSA phase and in ^{2}D on the IAM phase, to allow satisfactorily orthogonality, which implies that

342 the separation mechanisms used in each dimension are independent of each other [36].

343 First of all, we had to verify that the given (re-)equilibration volume in the ^{2}D separation was

344 suitable to achieve reproducibility in both retention time and chromatographic peak profile. To

find out this the separation was studied in ^{1}D and the same chromatographic conditions described

in 2.4.2 were applied, but the flow rate was halved and consequently broadened the separation

window by two times. This was done to allow assessment of even small discrepancies in the

chromatographic signals by visual inspection. The experimental procedure is properly detailed in

2.0 of the supporting information.

In brief, both the conditions detailed in 2.4.2 and that discussed in 2.5.2 for the first 36 minutes of

the mixed gradient elution program were applied. For the sake of conciseness, only the

chromatograms achieved by applying the former are discussed, but the results obtained were

highly comparable. The column was equilibrated by flushing 50.0 mL, *i.e.* 20 column volumes, at

constant flow rate, *i.e.* 2.5 mL min^{-1} of the starting mobile phase and then three different samples

(carbamazepine, desipramine, naproxen) and one sample mixture (acetaminophen, antipyrine,

carbamazepine and naproxen) were injected for four consecutive times. Figure 1 shows excellent

reproducibility of the measurements over the four runs and demonstrate that only 2.5 mL, *i.e.*

only one column volume, is sufficient to allow complete column re-equilibration. This most

probably takes place because the overall variation in the acetonitrile concentration is only 30%

(v/v) in both the elution programs. However, it is worth noting that while conventional LC

protocols recommend to equilibrate columns by flushing from 10 to 20 column volumes it is

common practice in LCxLC to significantly reduce this [37]. Lower equilibration volumes (0.5-1.0-

1.5 and 2.0 mL were tested) led to unreproducible results, with not overlappable signals and

compromised peak symmetry (data not shown). Consistently, Venter and co-workers used only 1.6

column volumes for re-equilibration but the gradients applied spanned from totally aqueous (with

0.1% v/v formic acid) to 100% acetonitrile [38].

Despite the potential of stationary phase components such as silica to engage nonspecific

secondary interactions, most reports [39] indicate that immobilized HSA conserves the binding

369   properties of the protein in solution, allowing fast and reliable analyses of binding interactions.

370   However, a further complication lies in the aspect that one or more drug molecules can in

371   principle compete for the same biding site, producing potentially diverse retention times when a

372   compound is analysed alone or in mixture with one or more competitive displacers [40]. Although

373   partial and allosteric competitions have been studied with HSA HPLC, a complete displacement has

374   never been reported [39]. However, a noticeable example of this is the diazepam/ibuprofen co-

375   binding [41]. To avoid any possible co-binding, we took the following arrangements: all the solutes

376   were injected individually and no retention time shifts were recorded when they were instead

377   injected in the same mixture and a competitive binding and HSA Site I occupancy assessment with

378   10 model drugs and an allosteric binder, *i.e.* (±)-warfarin, was run before each biomimetic

379   measurement to investigate about possible competitive binding with Site I of the HSA and rate of

380   occupancy of this enantioselective site.

381

382   **3.2 Competitive binding and HSA Site I occupancy assessment**

383

384   The results of this assessment are shown in Figure 2. Clearly, except for the most polar

385   compounds, *i.e.* acetaminophen and antipyrine, which overlap, a good orthogonal separation is

386   achieved for all the assayed solutes. Evidently, (+)-warfarin and (-)-warfarin display a different

387   interaction toward the HSA phase, being the former less retained than the latter. Analogously,

388   another acidic racemate, *i.e.* (±)-ketoprofen, is resolved in its enantiomers. These results go well

389   with the studies conducted by Zou and co-workers [42], which determined the stereoselective

390   binding of warfarin and ketoprofen to HSA by both microdialysis and 1D HSA HPLC. The authors

391   concluded that the S-enantiomers ((+)-ketoprofen and (-)-warfarin) bind to the HSA more strongly

392   than (R)-enantiomers to HSA and that HSA exhibit stronger stereoselectivity to warfarin than to

393   ketoprofen racemates [42]. This is in full agreement with our findings as the resolution of the (±)-

394   warfarin is evidently superior to that of (±)-ketoprofen signals, as can be inferred from Figure 2.

395   Consistently, the IAM phase exploited in ^{2}D did not allow any chiral separation and both

396   enantiomers exhibited same IAM affinity. Although IAM.PC phases do feature an asymmetric

397   carbon atom in their structure, they have never been reported to act as chiral selectors [16].

398

399   **3.3 2D LC bioaffinity experiments**

400

401   The first 2D LC bioaffinity experiment is visualized in Figure 3. While no refocusing issues can be

402   observed, the applied chromatographic conditions in both dimensions evidently allowed poor

403   separation of roughly a third of the dataset (compounds 1-10). These are extremely polar

404   compounds (-4.30 ≤ log P^N ≤ 0.53), whose interaction with both stationary phases is rather limited.

405   The least retained compound on both dimensions was enalaprilat (log k^{HSA} = -0.886 and log k^{IAM} = -

406   2.332), the compounds exhibiting the strongest affinity were (+)-ketoprofen on the HSA (log k^{HSA} =

407   1.246) and desipramine on the IAM (log k^{IAM} = 0.653) phases. This is consistent with previous

408   studies regarding both the selectivity of the IAM phase [43] and that of HSA [44]. Three are indeed

409   the most lipophilic bases included in the dataset, *i.e.* desipramine (pKa = 10.40), propranolol (pKa

410   = 9.45) and verapamil (pKa = 8.92)[45]. Among these, desipramine is the one featuring the highest

411   [cationic specie]/[neutral specie] ratio at pH 5, and, in agreement with the "pH piston hypothesis",

412   is the one having the strongest affinity with the IAM phase. Two are instead the acids

413   characterized by highest lipophilicity, *i.e.* fluvastatine (pKa = 5.50[46]) and ketoprofen (pKa =

414   4.45[45]). Again, between these, the analyte with a greater dissociation constant was retained for

415   a longer time, thus confirming the affinity of the HSA phase for acidic solutes. Clearly, no

416   breakthrough or peak refocusing issues are observable. As expected, chiral separation occurred

417   preferably for acidic racemates, *i.e.* (±)-fexofenadine, (±)-fluvastatin and (±)-ketoprofen, for which

418   anyhow the HSA matrix is known to have stronger affinity. However, although no chiral resolution

419   was achieved for the basic racemates (±)-atenolol, (±)-metoprolol and (±)-propranolol, the (±)-

420   verapamil racemate was instead enantioselectively retained to some extent. Interestingly, even if

421   a fluvastatin/salicylic acid competitive binding to the HSA has been reported by Tse *et al.* [47], no

422   variation in the retention factors was recorded when these compounds were individually

423   measured. Furthermore, Jattinagoudar and co-workers claimed that according to spectroscopy

424   and molecular docking-based studies they carried out [48], the secondary structure of serum

425   albumin was changed in the presence of fexofenadine. In our experiments again no variation in

426   the chromatographic retention coefficient was appreciated, therefore if any structural

427   rearrangement took place, this had apparently a negligible influence on the bioaffinity

428   measurements. Indeed, the authors studied in interaction of fexofenadine with bovine serum

429   albumin (BSA) and not with HSA. Even though these two proteins share 76% sequence homology,

430   they however differ due to the presence of two tryptophan residues in BSA, with Trp-213 located

431   within a hydrophobic binding pocket in the subdomain IIA, and Trp-134 located on the surface of

432   the albumin molecule in subdomain IB [49]. Moreover, Jattinagoudar and co-workers conducted

433   fluorescence spectroscopy binding measurements by using relatively high fexofenadine levels, *i.e.*

434   5-45 µM, to react with 2.5 µM BSA [48]. This is (at least) 50 times higher than the concentrations

435   applied in HSA based HPLC.

436   As said, although a good and orthogonal separation was achieved for most of the assayed

437   compounds, this method failed in discriminating the most polar molecules. Conceivably, these

438   feature remarkably diverse P_{eff} values, consequently we decided to apply a segmented gradient

439   elution program in ^{2}D with the aim of broadening the separation window of the compounds

440   elution within the first 36 minutes.

441   The result of this further method development are shown in Figure 4. Evidently, the compounds

442   eluting within the first 36 minutes are much better distributed than before over the separation

443   space. Again a part from cyclosporine A, whose peak shape suffers from some fronting, the

444   majority of analytical signals have good peak shape, with carbamazepine, hydrochlorothiazide and

445   losartan featuring excellent symmetry. An inversion in the elution order is seen for antipyrine and

446   atenolol (compound 10 and 11), being the latter more retained in eluents richer in aqueous buffer.

447   Although this circumstance might be unexpected when LC is exploited on electrically neutral

448   stationary phases, this is instead reasonable when we consider that IAM phases are instead

449   electrically charged and exhibit preferential affinity for cations [18]. Both antipyrine and atenolol

450   are bases, however the first (pKa = 6.8[29]) is much weaker than the second (pKa = 9.6 [29]).

451   Indeed, ionization is enhanced in fully aqueous phases as the addition of an organic modifier

452   lowers the dielectric constant of the medium. A possible explanation of this chromatographic

453   behaviour is that in pure aqueous eluent atenolol dissociates into its ions to a greater extent than

454   antipyrine, and its cations are more abundant at the experimental pH, *i.e.* 5.0, than its neutral

455   microspecies. However, when the starting mobile phase features already 25% (v/v), ionization

456   might occur to a lower extent and the contribution electrostatic and hydrophobic forces in the

457   whole separation mode is plausibly unbalanced slightly in favour of the latter. With this regards,

458   *n*-octanol/water lipophilicity of the neutral species of antipyrine (log P^N) and of the distributions at

459   both pH 5.0 and 7.4 (log $D^{5.0}$ and log$D^{7.4}$) is greater for antipyrine than for atenolol.

460

461   **3.4 Biopharmaceutical implications**

462

463   So far, we have discussed the separation only from an analytical point of view, however much

464   attention has been paid in developing an analytical method that allowed the affinity index to

465   retain relevance also from a biopharmaceutical perspective. From Figure 5, it is evident that all the

466   compounds located in the same area (blue frame) have intestinal effective permeability higher

467   than $1 * 10^{-4}$ cm $*$ s^{-1} , except for furosemide and (±)-fexofenadine, behaving as outliers. Actually,

468   furosemide was reported to be a substrate of a saturable active efflux transport system [50].

469   Consistently, the basolateral-apical apparent permeability of (±)-fexofenadine, estimated in Caco-2

470   monolayers, markedly reduced in the presence of increasing concentration of P-glycoprotein

471   inhibitors, suggesting that this efflux protein carrier is the main transport route for this

472   therapeutic [51]. The active uptake of xenobiotics requires ATP hydrolysis and occurs via specific

473   recognition of structural motifs that both the exploited separation modes cannot possibly depict.

474   However, it is relevant that all the compounds having intestinal absorption greater than a

475   threshold – except the two analytes above discussed – populate a specific region of the 2D plot.

476   We are aware that the size of the dataset may be relatively limited, however as already

477   highlighted in the introduction, these intestinal effective permeability experiments are hardly

478   performed due to ethical reasons and the criticism these human testing methodologies are

479   increasingly facing from the general public. Additionally, pooling biological activity data from

480   different sources might then transpire in a misleading exercise in the light of the aspect that

481   significant interlaboratory variability (sometimes even higher than 80%) has occasionally been

482   reported [52]. For this reason, we decided to select biological activity data from a single

483   bibliographic source. Moreover, one of the advantages of this screening platform is that the

484   results can be evaluated by simple visual inspection, while statistical modelling often requires

485   technical expertise and a sound background to be properly assessed.

486   However, some conventional modelling by calculating the affinity indexes on each dimension by

487   using Eq. (2) was still envisaged. We took into account the separation displayed in Figure 3 and

488   conducted by applying the conditions detailed in 2.5 because it is necessary that the bioaffinity

489　indexes are achieved in the same thermodynamic conditions to allow fair comparison. The affinity

490　data in each dimension, along with log P^N, log $D^{7.4}$, clog $D^{5.0}$, P_{eff} values and supplier are reported

491　in Table 1. Chemical structures are instead reported in Table S2. Interestingly, when the

492　partitioning values were plotted against biological data, no significant relationship was achieved in

493　all cases (Figure 6).

494　However, 3D scatterplots studying both HSA and IAM bioaffinity *vs* "classical" *n*-octanol/water

495　partition coefficients of the neutral species and of the neutral/ionized distribution both at blood,

496　*i.e.* 7.4, and at duodenum pH, *i.e.* 5.0 were then generated. These 3D scatterplots are represented

497　in Figure 7 and (B) and (C) could be considered as a simulation a 3D LC experiments in in which the

498　3D is exploited on a neutral hydrophobic phase, such as C18 or C8, and the aqueous eluent are

499　buffered at pH 7.4 and 5.0 respectively. In fact, retention on stationary phases based on

500　hydrocarbons has been proved to be driven by the lipophilicity of the distribution coefficients at

501　the experimental pH, although some secondary interactions can take place due to the free silanol

502　groups [53]. A further complication is that the *in vivo* intestinal mucosa features various degrees of

503　leakiness as a consequence of the different expression of tight junctions and this may allow some

504　paracellular passage. Pearce *et al.* demonstrated that the expression of certain tight junction

505　proteins varied with cell type, with occludin and tricellulin levels being high in both intestinal stem

506　cells (ISCs) and Paneth cells, and claudin-1, -2, and -7 expression being enhanced in Paneth cells,

507　ISCs, and enterocytes, respectively [54]. The paracellular passage pathway is likely for small

508　hydrophilic molecules, having sizes compact enough to slip through the cell-to-cell gaps. To

509　distinguish any possible involvement of paracellular passage, we deliberately split our dataset in

510　two subsets: compounds having mass weight (MW) inferior than 200 Da, for which a relevant

511　contribution of paracellular passage can be reasonably hypothesized, and analytes heavier than

512　200 Da, for which a pure transcellular migration is instead highly likely. Recent scientific reports

513   [55] suggest that molecular size and O plus N atom count and OH plus NH count of newly designed

514   drugs are steadily but constantly increasing, while molecular lipophilicity has remained relatively

515   unchanged. Therefore, since it might be challenging for pharma chemists dealing with rational

516   drug design to comply to a 200 Da MW constrain, predicting passive transcellular permeability is

517   much more valuable, especially if we consider that there might be a huge variation in the degree

518   of leakiness of intestinal cells among individuals. While plots (B) and (C) show a quite

519   homogeneous distribution of the subsets, interestingly most of the compounds having fair and

520   good effective intestinal permeability populate a specific area of plot (A), displayed in a red

521   square, with the exception of one datapoint, *i.e.* furosemide. The characteristics of this analytes

522   have already been discussed above.

523   Although further studies should be undertaken, these results seem extremely relevant and might

524   assist – alone or in combination with other methodologies – pharmaceutical chemists in screening

525   therapeutics for their intestinal absorption potential without sacrificing/distressing animals and/or

526   running complex and sometime resource-intensive statistical modelling.

527

528

529   **4.0 Concluding remarks**

530

531   A 2D comprehensive LC-based biomimetic platform has been for the first time developed and

532   validated as a screening tool for drug diffusion studies. The separation modes were based on

533   biological structures that are crucial for both drug absorption and distribution processes.

534   Specifically, a stationary phase embedding HSA, the most abundant plasma protein in humans,

535   was exploited in ^{1}D. An IAM phase, aimed at mimicking the lipoidal composition of plasma

536   membranes, was instead operated in ^{2}D.

537   This set-up allowed a good and orthogonal separation of 30 model drugs, whose P_{eff}, assayed on

538   humans, was known from a single bibliographic source. Moreover, if we except two compounds

539   whose uptake is protein carrier- mediated, all the compounds populating the same area of the 2D

540   plots have P_{eff} greater than than $1 * 10^{-4}$ cm $* s^{-1}$. This implies that in order for a drug to be

541   successfully up taken, its affinity for these crucial biostructures has to lie in specific affinity ranges.

542   In addition, these bioaffinities were also studied *vs* classical *n*-octanol/water partitioning data and

543   visualized in 3D scatterplots. When the logarithm of the *n*-octanol/water partition coefficient of

544   the neutral species is plotted on the z axis (and HSA and IAM values on the x and y axis), again the

545   solutes having favourable P_{eff} and – for which an involvement of any paracellular passage

546   contribution is rationally unlikely – concentrate in the same region of the plots.

547   We acknowledge that these must be seen as preliminary results and further studies are needed to

548   further confirm this evidence. However, we also believe this design paves an entirely new way to

549   profile pharmaceutically relevant compounds for their *in vivo* absorption and distribution

550   potential.

551

Table 1

Compound	log k^{HSA}	log k^{IAM}	log P^N	log $D^{7.4}$	clog $D^{5.0}$[30]	P_{eff}[31] $(10^{-4} cm \cdot s^{-1})$	Supplier
acetaminophen	-0.221	-1.487	0.34[28]	0.34[28]	0.34[29]	1.76	Merck
amiloride	0.034	-0.320	-0.26[28]	-1.53[28]	-0.12	1.63	TCI
antipyrine	0.000	-0.403	0.56[28]	0.26[28]	1.61	4.45	TCI
(±)-atenolol	-0.221	0.294	0.22[28]	-2.01[28]	-3.31	0.21	Merck
carbamazepine	0.833	0.204	2.45[28]	2.45[28]	3.22	4.30	Merck
cimetidine	0.000	0.226	0.48[28]	0.34[28]	-1.27	0.44	Merck
creatinine	-0.167	-0.934	0.54[30]	0.53[30]	-0.05	0.30	Merck
cyclosporin A	0.415	0.632	1.40[29]	3.38[30]	3.38	1.63	TCI
desipramine	0.914	0.653	3.79[28]	1.38[28]	0.05	4.45	Merck
enalapril	-0.221	-1.218	0.16[28]	-1.75[28]	0.38	1.57	TCI
enalaprilat	-0.886	-2.332	-0.13[28]	-2.74[28]	-2.27	0.20	Sanbio
(+)-fexofenadine	1.108	0.184	5.68[30]	2.48[30]	2.45	0.47	Sanbio
(-)-fexofenadine	1.057						
(+)-fluvastatin	1.165	0.219	4.17[28]	1.14[28]	3.27	2.38	Sanbio
(-)-fluvastatin	1.128						
furosemide	1.094	0.002	2.56[28]	-0.24[28]	0.84	0.25	Acros
(+)-griseofulvin	0.893	0.331	2.20[28]	2.20[28]	2.20[29]	1.14	Acros
hydrochlorothiazide	0.532	0.043	-0.03[28]	-0.18[28]	-0.16	0.12	TCI
(+)-ketoprofen	1.246	0.145	3.16[28]	-0.11[28]	2.31	8.45	Merck
(-)-ketoprofen	1.226						
lisinopril	-0.301	-1.218	-1.01[29]	-4.30[30]	-4.25	0.33	Sanbio
losartan	1.017	0.422	1.19[29]	4.03[30]	5.24	1.14	Sanbio
L-phenylalanine	-0.398	-1.487	-1.38[29]	-1.67[30]	-1.67	4.07	Merck
(±)-metoprolol	0.000	0.380	1.95[28]	-0.24[28]	-2.08	1.16	Merck
nifedipine	0.881	0.555	3.17[28]	3.17[28]	3.17[29]	4.40[32]	Sanbio
piroxicam	1.153	0.087	1.98[28]	0.00[28]	0.56	7.06	Sanbio
(±)-propranolol	0.764	0.596	3.48[28]	1.41[28]	-1.07	2.82	Merck
ranitidine	0.205	0.373	0.45[28]	-0.53[28]	-1.87	0.37	Merck
salicylic acid	1.000	-0.485	2.19[28]	-1.68[28]	-0.27	2.67	Merck
terbutaline	0.069	-0.699	-0.08[28]	-1.35[28]	-2.40	0.30	Merck
valacyclovir	-0.699	-1.487	-0.30[29]	-1.26[30]	-3.35	1.66	Sanbio
(+)-verapamil	0.820	0.342	4.33[28]	2.51[28]	0.79	6.18	Merck
(-)-verapamil	0.858						6.21	

561   **Figure 1.**
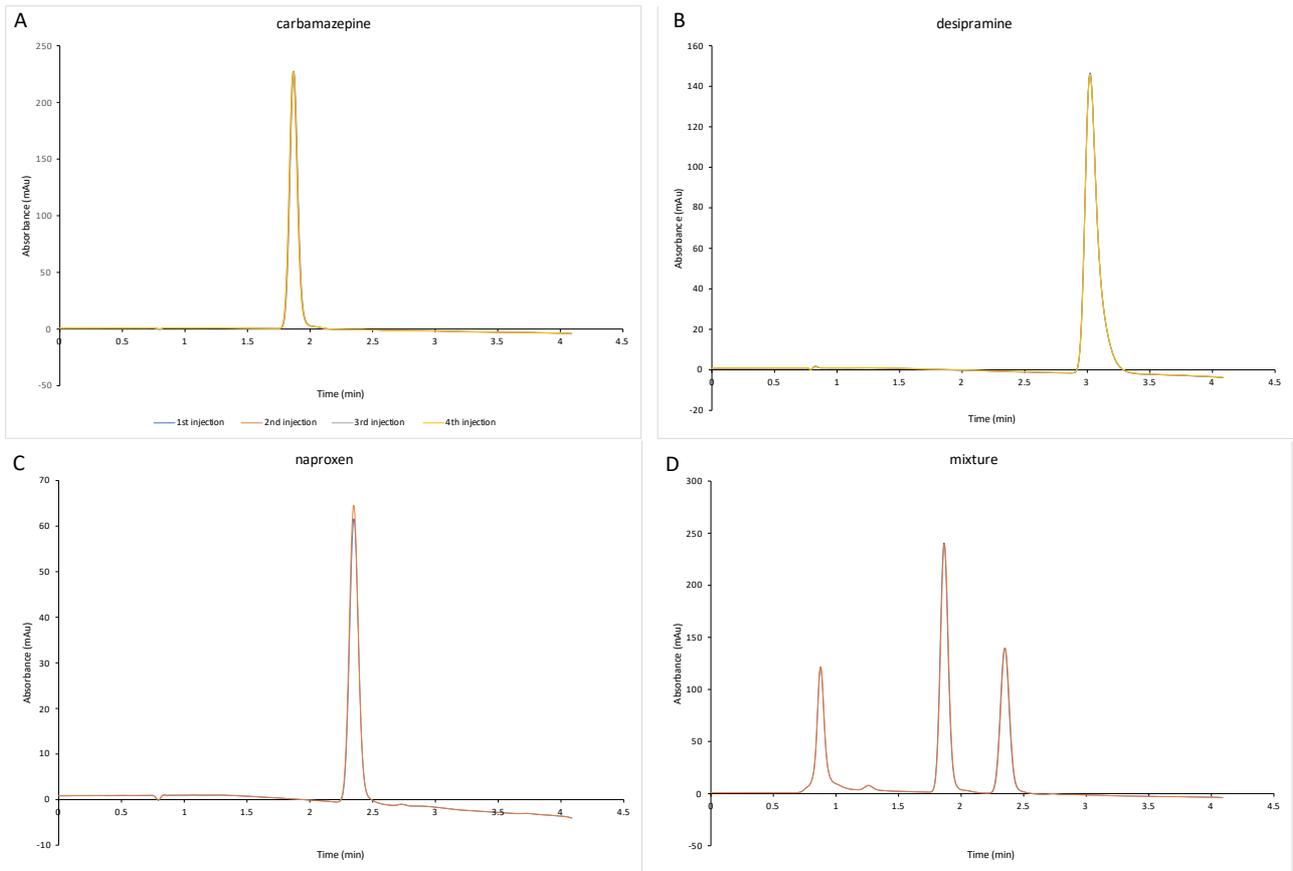
Figure 2
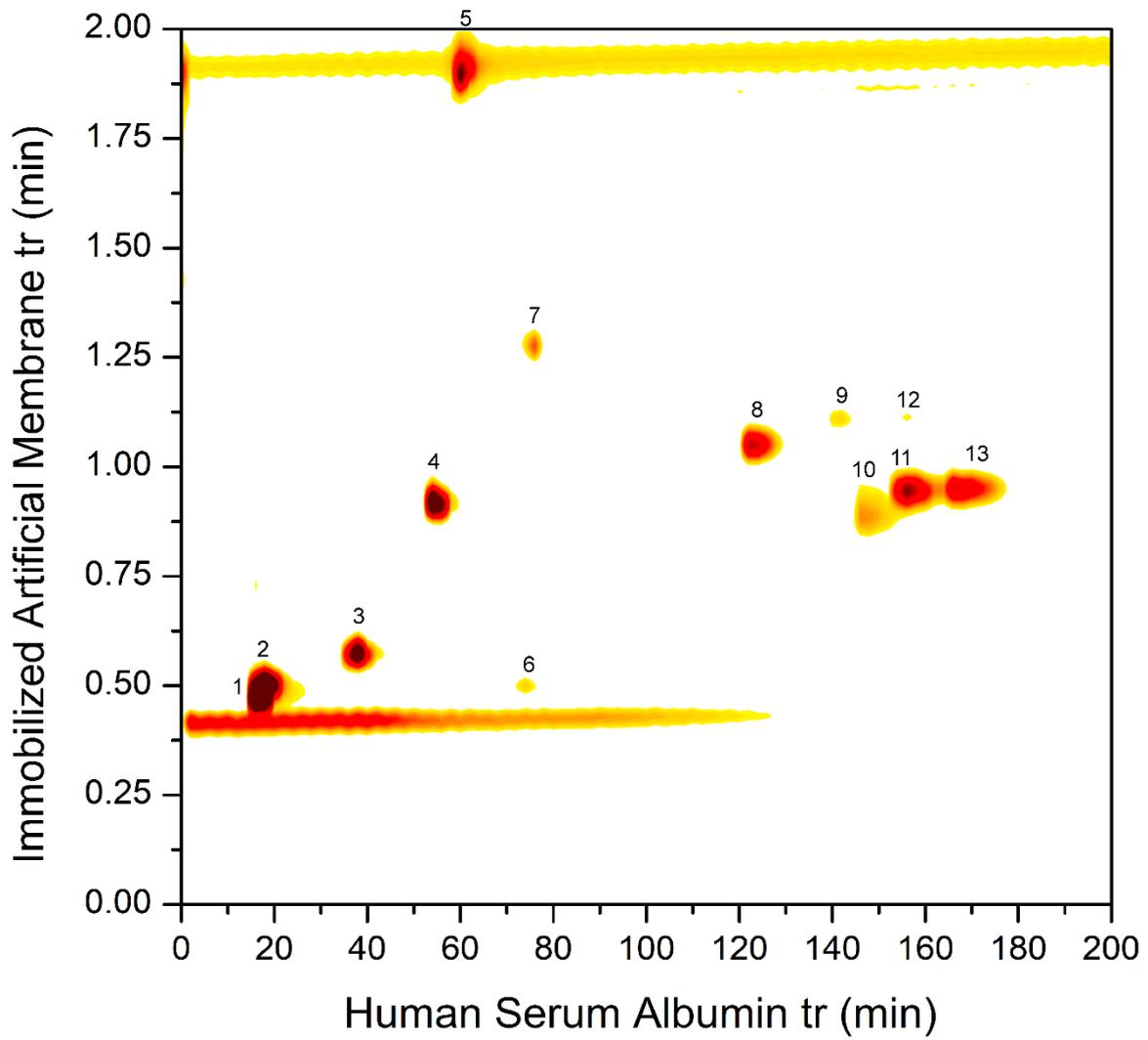
1. acetaminophen
2. antipyrine
3. hydrochlorothiazide
4. carbamazepine
5. desipramine
6. salicylic acid
7. nifedipine
8. losartan
9. (+)-warfarin
10. piroxicam
11. (-)-ketoprofen
12. (-)-warfarin
13. (+)-ketoprofen

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605   **Figure 3**

1. acetaminophen
2. antipyrine
3. lysinopril
4. amiloride
5. enalaprilat
6. L-phenylalanine
7. creatinine
8. valacyclovir
9. enalapril
10. terbutaline
11. cimetidine
12. (±)-atenolol
13. (±)-metoprolol
14. ranitidine
15. cyclosporin A
16. hydrochlorothiazide
17. (±)-propranolol
18. (+)-verapamil
19. (-)-verapamil
20. carbamazepine
21. nifedipine
22. (+)-griseofulvin
23. desipramine
24. losartan
25. salicylic acid
26. (-)-fexofenadine
27. furosemide
28. (+)-fexofenadine
29. (-)-fluvastatin
30. piroxicam
31. (+)-fluvastatin
32. (-)-ketoprofen
33. (+)-ketoprofen
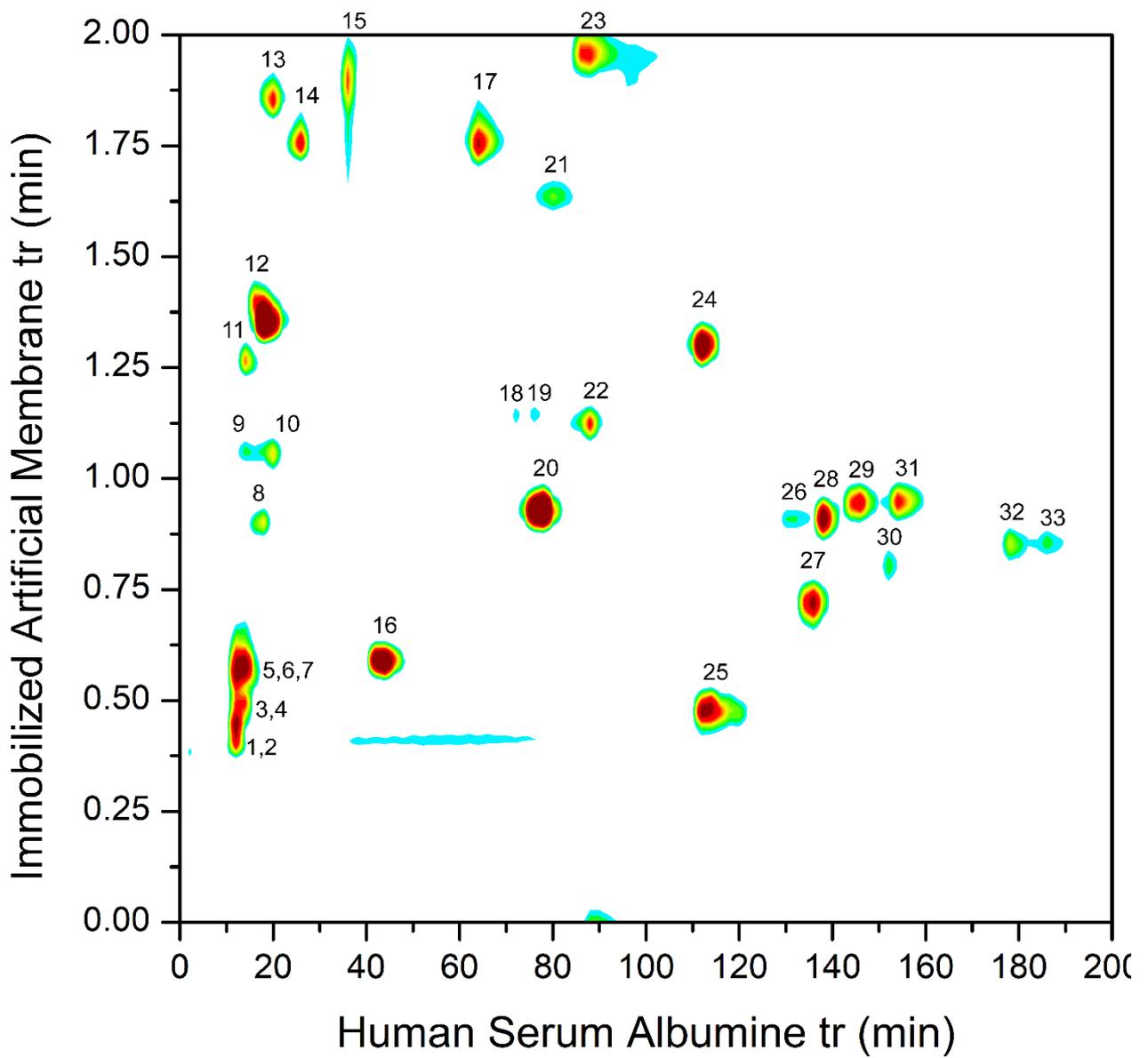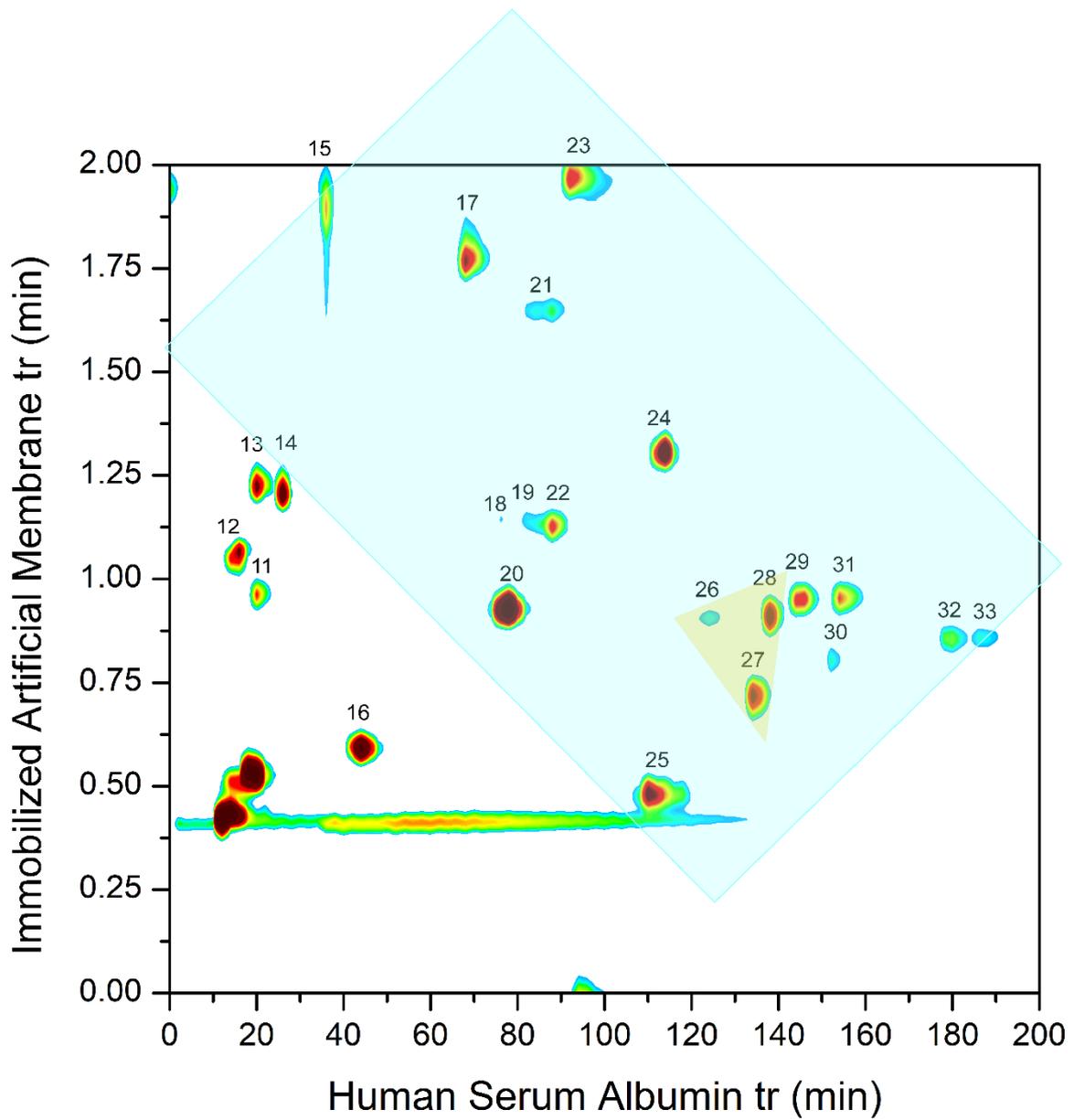
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607   **Figure 4**

Figure 5

621 **Figure 6**
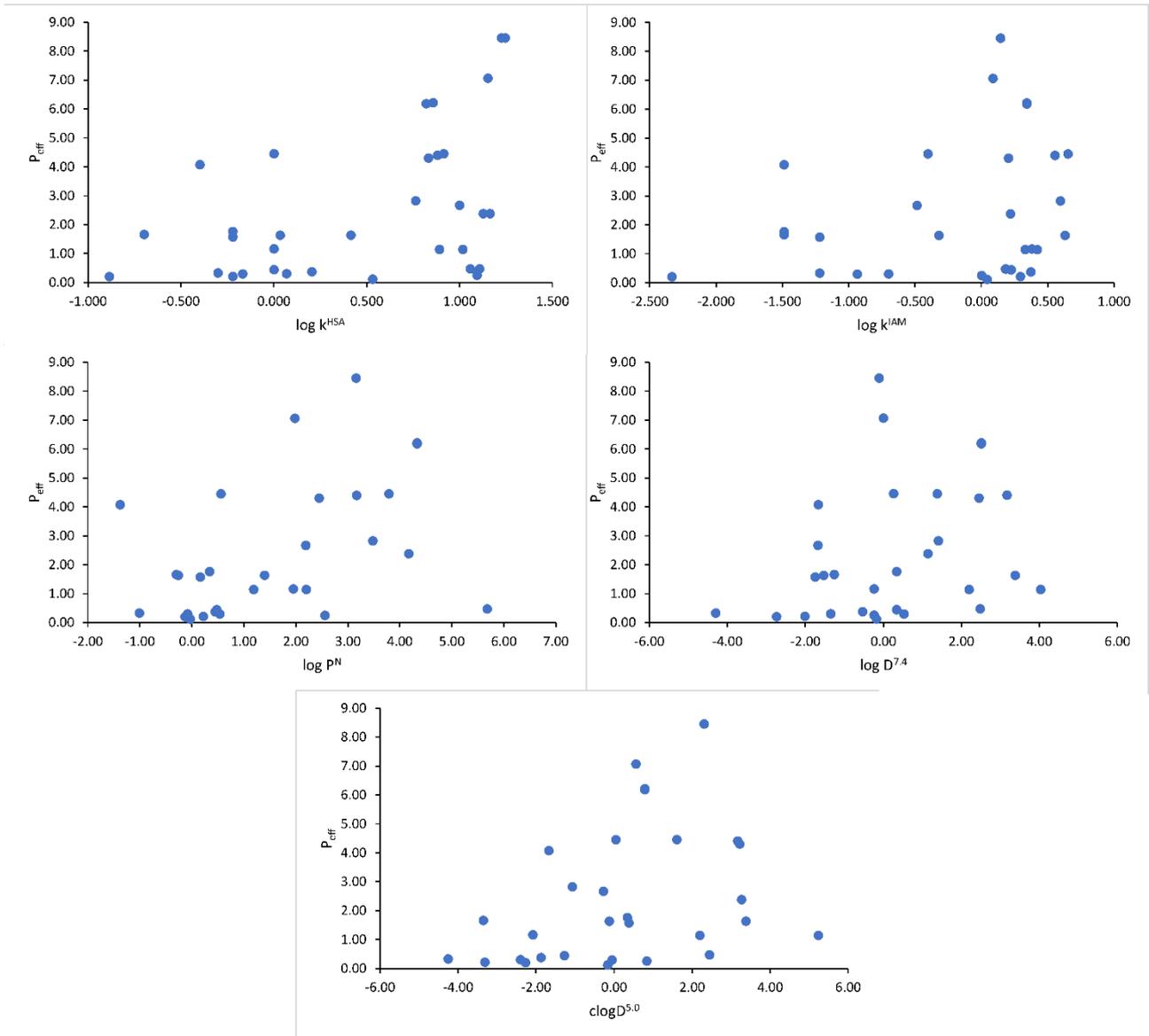
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631   **Figure 7**
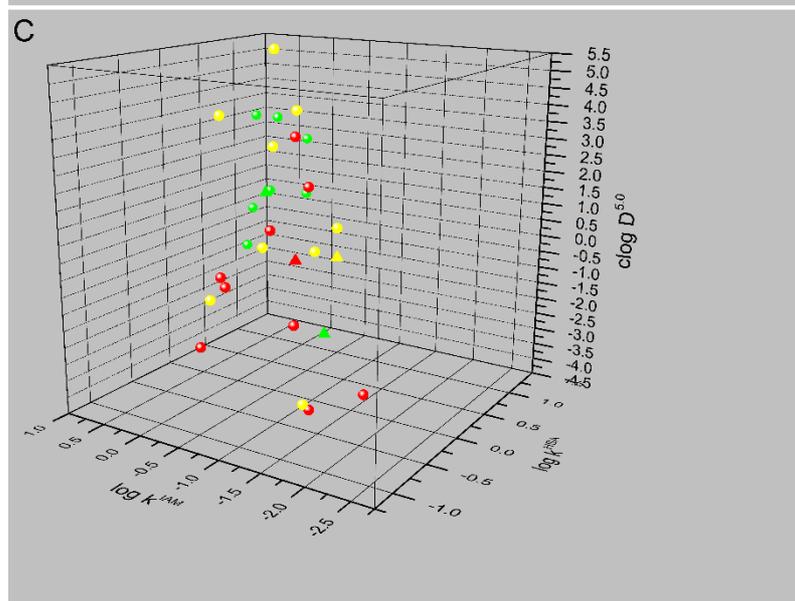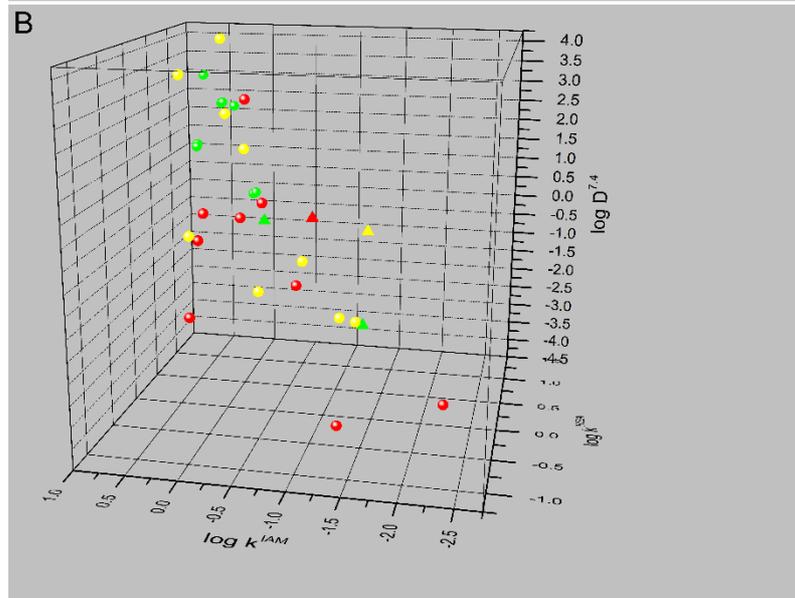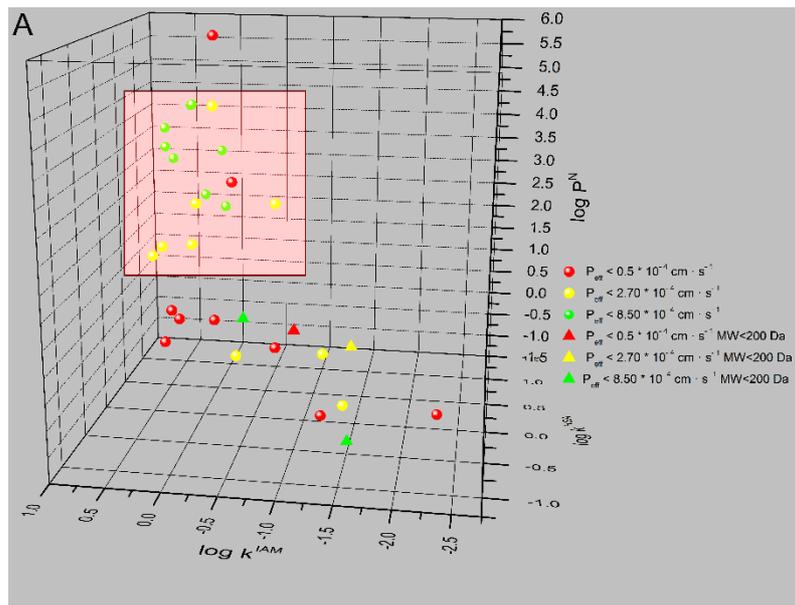
Captions

Table 1. Logarithms of chromatographic retention coefficients on the HSA (log k^{HSA}) and IAM (log k^{IAM}) stationary phases, of the n-octanol/water partition coefficients of the neutral forms of the analytes (log P^N), of the distribution coefficients at 7.4 (log $D^{7.4}$) and 5.0 (clog $D^{5.0}$) pH, intestinal effective permeability values ($* 10^{-4} * cm * s^{-1}$) and supplier of the considered analytes.

Figure 1. IAM chromatograms showing four consecutive runs performed after flushing 20 column volumes. A 25 µg mL^{-1} solution of the compounds carbamazepine (A), desipramine (B), naproxen (C) and a mixture of acetaminophen, antipyrine, carbamazepine and naproxen (D) was injected. Further details are reported in 2.0 of the supporting information.

Figure 2. Contour plot at 254 nm obtained for the HSA × IAM separation of a test mixture for competitive binding and HSA Site I occupancy assessment purposes. Further experimental details are reported in 2.4 of the main body of the manuscript.

Figure 3. Contour plot at 220 nm obtained for the HSA × IAM separation of a dataset for bioaffinity measurements, in which the fixed gradient elution program is applied in ^{2}D for the whole duration of the run. Further experimental details are reported in 2.5 of the main body of the manuscript.

Figure 4. Contour plot at 220 nm obtained for the HSA × IAM separation of a dataset for bioaffinity measurements, in which a segmented gradient elution program is applied in ^{2}D. Further experimental details are reported in 2.5 of the main body of the manuscript.

657   **Figure 5.** Contour plot at 220 nm obtained for the HSA × IAM separation of a dataset for Bioaffinity

658   measurements, in which a mixed gradient elution program is applied in 2D. The region in which

659   compounds feature favourable intestinal effective permeability is represented in a blue square.

660   The outliers are instead displayed in a yellow triangle.

661

662   **Figure 6.** Scatterplots in which the intestinal effective permeability is studied *vs* the logarithms of

663   chromatographic retention coefficients on the HSA (log k^{HSA}, top left), IAM (log k^{IAM}, top right)

664   stationary phases, the *n*-octanol/water partition coefficients of the neutral forms of the analytes

665   (log P^N, middle left), of the distribution coefficients at pH 7.4 (log $D^{7.4}$, middle right) and 5.0 pH

666   (clog $D^{5.0}$, bottom).

667

668   **Figure 7.** 3D scatterplot studying both HSA and IAM affinities vs the *n*-octanol/water partition

669   coefficient of the neutral forms of the analytes (log P^N), of the distribution coefficient at 7.4 (log

670    $D^{7.4}$) and 5.0 (clog $D^{5.0}$) pH. Compounds with mass weight lower than 200 Da are pictured in

671   triangles, whereas those heavier than 200 Da are circle shaped. The colors of the datapoints refer

672   to a good (green), medium (yellow) and poor (red) extent of intestinal effective permeability.

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