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1 **Purification of dirucotide, a synthetic 17-aminoacid**
2 **peptide, by ion exchange centrifugal partition**
3 **chromatography**

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27 **Purification of a 17-aminoacid peptide called dirucotide**
28 **using ion exchange centrifugal partition chromatography**

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37

38 **Abstract:** Dirucotide is a synthetic drug candidate for the treatment of multiple
39 sclerosis. This 17-aminoacid peptide was successfully purified by ion exchange
40 centrifugal partition chromatography. The optimized conditions involved the biphasic
41 methyl *tert*-butyl ether/acetonitrile/*n*-butanol/water (2:1:2:5, v/v) solvent system in the
42 descending mode, the di(2-ethylhexyl)phosphoric acid cation-exchanger with an
43 exchanger (di(2-ethylhexyl)phosphoric acid)/dirucotide mole ratio of 100 and Ca²⁺
44 ions in aqueous solution as displacer. Critical impurities were efficiently eliminated
45 and dirucotide was recovered in high yield and purity (69 % and 98 %, respectively)
46 and with a productivity of 2.29 g per liter of stationary phase per hour.

47

48 **Keywords:** Bioactive peptide, dirucotide, Centrifugal Partition Chromatography, Ion
49 Exchange, multiple sclerosis.

50

51 **1. Introduction**

52 Multiple sclerosis (MS) is an inflammatory neurodegenerative disease in which the
53 myelin sheath around the axons of the brain and spinal cord are damaged. [1] MS
54 affects young adults and it is more common in women. Few therapeutic agents such
55 as interferon β -1a and β -1b, natalizumab,— mitoxanthrone, tereflunomide,
56 alemtuzumab, dimethylfumarate [2-4] have been approved by regulatory agencies to
57 treat MS.

58 Other drugs, such as dirucotide (figure 1), are under evaluation.[5] This synthetic
59 17-aminoacid peptide, also called MBP8298 and co-developed by BioMS Medical
60 and Eli Lilly Company is an analog of myelin basic protein with a sequence
61 corresponding to its 82-98 residues.[6] First stages of clinical trials have
62 demonstrated the high potential of this drug for secondary progressive multiple
63 sclerosis treatment [3,7] but the result of final phases were not satisfactory, due to
64 the lack of efficacy although dirucotide was generally well tolerated. Dirucotide
65 analogues such as citrullinated modified peptide analogues are currently under
66 development. ~~Dirucoted~~ Dirucotide was produced by solid phase synthesis and the
67 last purification steps appeared to be the bottleneck of the process: some critical
68 impurities were partially co-eluted in classical chromatographic processes, such as
69 reversed phase High Performance Liquid Chromatography (HPLC).

70

71

Insert here figure 1

72

73 In order to improve the final purification of dirucotide, other chromatographic
74 processes were investigated such as support-free liquid/liquid chromatography and
75 particularly Centrifugal Partition Chromatography (CPC) [8,9], this technique having
76 emerged as an interesting alternative tool for the purification of natural and synthetic
77 products. For instance, the support-free liquid-liquid chromatography, in its
78 hydrostatic (CPC) and its hydrodynamic (Countercurrent Chromatography or CCC)
79 variants [8], has already been applied to peptide purification. In this context, specific
80 biphasic solvent systems were developed for the purification of short or hydrophobic
81 peptides in the elution mode using, for example, the quaternary biphasic system
82 MtBE/acetonitrile/*n*-butanol/water with 1% TFA. [10-12]

83 In addition to the common elution mode, the displacement mode was introduced in
84 countercurrent chromatography (CCC) in 1994 [13] and very early applied to the
85 purification of protected peptides.[14,15] Displacement mode in CPC encompasses
86 two types of processes: pH-zone refining and ion-exchange. [16-18] This is a highly
87 productive and selective purification method that was successfully applied to very
88 diverse classes of organic molecules. The pH-zone refining mode, is dedicated to
89 solutes that show a dramatic difference in polarity, and therefore in solubility,
90 between their neutral and ionized forms. This feature excludes the application of pH-
91 zone-refining to ionized, or strictly water-soluble, or to amphoteric molecules such as
92 free peptides. More recently, an efficient purification process was reported for the
93 purification of unprotected peptides by strong ion exchange CPC (IX-CPC). [18,19] It
94 makes use of a very common and cheap lipophilic cation exchanger, di-(2-
95 ethylhexyl)phosphoric acid (DEHPA), whose anionic sites are activated by addition of
96 triethylamine (TEA). The activated exchanger is dissolved in the organic stationary
97 phase in order to extract the hydrophilic peptides by ionic association during the

98 injection step. Then, a displacer-containing aqueous mobile phase is pumped
99 through the stationary phase, thus forcing the ionic analytes to be displaced in the
100 aqueous mobile phase and to move along the column. A Ca^{2+} aqueous solution was
101 used for this purpose in the present case. As in any other displacement
102 chromatography techniques, the analytes move in the CPC column as an isotachic
103 train, with the ionization state of the analytes being left unchanged throughout the
104 whole chromatographic process. This last point constitutes the main difference
105 between ion exchange and pH-zone refining, which involves multiple changes in the
106 ionization state of the analytes through acid-base reactions.

107

108 In addition to dipeptides [18], IX-CPC was also applied to the purification of natural
109 products such as glucosinolates, [20] and organic acids [21] for instance. In all cases,
110 it demonstrated ease of implementation as well as high selectivity and productivity.

111 Then, the main aim of this work was to assess the performance of the IXPCP
112 strategy for the purification of a peptide active pharmaceutical ingredients such as
113 dirucotide.

114

115 **2. Material and Methods**

116 *2.1. Reagents*

117 Acetonitrile (CH_3CN), *n*-butanol (*n*-BuOH), methyl-*tert*-butyl ether (MtBE), methanol
118 (MeOH) and deuterated chloroform (CDCl_3) were purchased as chromatographic
119 grade solvents from Carlo Erba (Rodano, Italy). Trifluoroacetic acid (TFA), calcium
120 chloride (CaCl_2), di(2-ethylhexyl)phosphoric acid (DEHPA), triethylamine (TEA) were
121 purchased from Acros Organics (Illkirch, France). Water was purified by de-ionization
122 and reverse osmosis. Dirucotide was kindly provided by Lonza (Braine, Belgium).

123 The HPLC purity of the sample of dirucotide (Lonza lot number 3AH2) was 84.3 %.
124 Dirucotide is a peptide containing 17 amino acids, with an isoelectric point of 7.5
125 (according to Emboss emboss.sourceforge.net) and a molecular weight mass of
126 2012 g.mol⁻¹. Its sequence is H₂N-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-
127 Ile-Val-Thr-Pro-Arg-Thr-COOH.

128

129 *2.2. CPC Apparatus*

130 The separations were performed on a FCPC Kromaton Technologies apparatus
131 (Annonay, France) using a rotor made of 20 circular partition disks (1320 partition
132 cells: 0.130 mL per cell; total column capacity: 205 mL, dead volume: 32.3 mL).
133 Rotation speed could be adjusted from 200 to 2000 rpm, producing a centrifugal
134 force field in the partition cell of ~120 g at 1000 rpm and 480 g at 2000 rpm. The
135 solvents were pumped by a Dionex P580HPG 4-way binary high-pressure gradient
136 pump (Sunnyvale, CA, USA).

137 The samples were introduced into the column through a preparative scale sample
138 injector 3725 (Rheodyne, Rohnert Park, CA, USA) equipped with a 10 mL sample
139 loop. Effluent detection monitoring was controlled by a Dionex UVD 170S detector at
140 $\lambda = 215$ nm equipped with a preparative flow cell (6 μ L internal volume, path length of
141 2 mm). Fractions (2 mL) were collected by a Pharmacia Superfrac collector (Uppsala,
142 Sweden). The experiments were conducted at room temperature (22 ± 2 °C).

143

144 *2.3. Preparation of biphasic solvent system for CPC runs*

145 Biphasic system (2 L) was prepared by mixing MtBE, CH₃CN, *n*-BuOH and water in
146 the suitable proportions in a separatory funnel. They were vigorously shaken and
147 then allowed to settle until the phases became limpid. After phase separation,

148 DEHPA was added to the organic stationary phase. TEA was then added to activate
149 DEHPA at the DEHPA/TEA molar ratio of 46.5. The mobile phase was prepared by
150 adding the appropriate amount of CaCl₂. The experimental conditions are presented
151 in table 1.

152

153 *2.4. Sample preparation for CPC runs*

154 100, 200 and 400 mg of dirucotide sample were dissolved in 7.5 mL of fresh aqueous
155 phase (without displacer). This aqueous solution was equilibrated with 2.5 mL of
156 DEHPA-free organic phase to restore saturation of the aqueous phase (about 1 mL
157 the organic phase dissolves in the aqueous phase).

158 1 g of dirucotide was dissolved in 15 mL of fresh aqueous phase, and this solution
159 was equilibrated with 5 mL of fresh organic phase to restore saturation of the
160 aqueous phase and thus the biphasic system. The two phases are then loaded
161 through the injection loop.

162

163 *2.5. CPC experimental conditions*

164 All experimental data are summarized in Table 1 and the overall protocol is shown
165 figure 2. Before any experiment, the column was washed by MeOH/water (50:50, v/v)
166 in the ascending mode (at 20 mL/min and 200 rpm). Then, two column volumes (410
167 mL) of organic stationary phase (SP) were pumped in the descending mode at the
168 same flow rate and rotation speed. The sample was then injected through the sample
169 loop at 2 mL/min in the descending mode, the rotation speed being increased to
170 1200 rpm. Afterwards, the displacer-free mobile phase (MP) (50 mL on average) was
171 pumped at 2 mL/min in order to allow column equilibration. Finally, the aqueous
172 mobile phase containing the displacer (Ca²⁺) was pumped at 2 mL/min, and the

173 fractions were collected every minute. The aqueous effluent was monitored by on-
174 line UV absorbance measurement at $\lambda = 220$ nm. Stationary phase retention and
175 back pressure were about 75 %. 35 bars, respectively. Fractions were lyophilized
176 after HPLC analysis to recover the dirucotide.

177

178 *Insert here table 1 and figure 2*

179

180 2.6. Fraction analysis

181 Quantification was performed on a customized Dionex Summit HPLC system,
182 equipped with a P580 pump, an ASI-100 automated injector, a STH column oven and
183 a UVD340S diode array detector and a Jupiter Proteo (250 x 4.6 i.d., 4 μ m particle
184 size) column equipped with Security Guard Analytical Guard cartridge system KJ0-
185 4282 with C12 4 x 3.0 mm cartridge (Phenomenex, Le Pecq, France). The mobile
186 phases were water 0.1% TFA (solvent A) and acetonitrile 0.09% TFA (solvent B).
187 The water/acetonitrile was set as follows: the starting composition was a mix solvent
188 A/solvent B (85:15, v/v). It was raised to 45% (v/v) acetonitrile in 30 min by applying a
189 linear gradient. Flow rate was 1 mL/min. UV detection was fixed at $\lambda = 220$ nm. The
190 temperature of the column oven was set at 40°C. All of the chromatographic data
191 management was ensured by the Chromeleon software 6.0.1 version (Dionex).

192 Dirucotide retention time was about 21 min, and critical impurities retention time were
193 20, 20.5 and 22.3 min (respectively impurities C, A and B, see figure 3). Purities are
194 expressed directly on the basis of UV absorbance of dirucotide and impurities on the
195 final pooled fractions.

196

197 *Insert here figure 3*

198

199

200

201 2.7. HPLC fractogram construction

202 A fixed volume of each fraction (10 μ L) was analyzed by HPLC, following the protocol
203 described section 2.6. Area under the curve (AUC) of each peak was plotted per time
204 to render the the HPLC fractogram.

205

206 3. Results and Discussion

207 Dirucotide is assembled by solid phase peptide synthesis starting from preloaded
208 Fmoc-Thr(tBu)-Wang resin and carrying out suitably protected amino acids for
209 Fmoc/tBu synthesis strategy (see electronic supplementary material for the complete
210 protocol). This solid phase synthesis of dirucotide generates peptidic impurities
211 (figure 3) and their level has to meet the final API specifications after purification.
212 Some impurities are critical if they partially co-elute with the main product or if they
213 have a significant impact on the purification yield. Therefore, various purification
214 processes such as elution or displacement CPC were considered, in which different
215 interactions between analytes and the stationary phase may occur. The first step of
216 this study was the selection of the more suitable chromatographic mode, namely
217 elution or displacement. Some biphasic solvent systems are already known for their
218 efficiency in elution mode peptide purification. These systems are compatible with
219 free and protected peptides.[7] Regarding the purification of dirucotide, only the
220 MtBE/CH₃CN/*n*-BuOH/water (2:1:2:5, v/v) system fulfilled both solubility and
221 dirucotide partition coefficient criteria, the latter being evaluated by using the shake
222 flask procedure and ninhydrin reagent for TLC visualization.

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223 As previously published, IX-CPC can be considered as a potentially effective
224 alternative to the elution mode for the preparative purification of small unprotected
225 peptides. [18,19] Indeed, this method was successfully applied to free dipeptide
226 purification [9] or capture and we proposed here to extend its scope to longer
227 peptides, such as the 17-aminoacid dirucotide. In this work, the aim is to evaluate the
228 interest of the technique for the purification of a large peptide, (*i.e.* a synthetic 17
229 amino-acid peptide).

230 Dirucotide isoelectric point is 7.5 and 7 cationic positions are generated in the
231 presence of DEHPA. These sites allow electrostatic interactions to take place
232 between dirucotide and anionic phosphate groups of the exchanger. The operating
233 conditions were adapted from our previous work on dipeptides.[18] They include
234 MtBE/CH₃CN/*n*-BuOH/water (2:1:2:5, v/v) as solvent system, DEHPA as exchanger,
235 TEA as DEHPA activator and Ca²⁺ (CaCl₂ in water) as strong displacer. The
236 [DEHPA]/[TEA] and [DEHPA]/[CaCl₂] ratios were respectively 46.5 and 10.4 and
237 calculated on the basis of the optimal experimental conditions that were previously
238 reported (Table 1).[18]

239 In IX-CPC, the exchanger/analyte ratio plays an important role, especially in the case
240 of peptides, because they may own many ionization sites. In our previous work, we
241 demonstrated that a exchanger/(ionized sites of the analytes) ratio comprised
242 between 5 and 30, depending on the chemical structure of the analyte, provide a
243 good separation.[9,10] Thus, a mole ratio $n_{\text{DEHPA}}/n_{\text{dirucotide}}$ of 100, corresponding to a
244 ratio $n_{\text{DEHPA}}/n_{\text{(positive charges of the analyte)}}$ ratio of about 25 was initially tested (Table 1).
245 The injected mass was set at 200 mg in preliminary purification tests. The displacer
246 (Ca²⁺) containing mobile phase was pumped 5 min after column equilibration. Indeed,
247 a washing step is generally applied at the beginning of the IXCPC run by pumping

248 the aqueous mobile phase without displacer for several minutes. The hydrophilic
249 non-ionic compounds are thus eluted before the displacement process. In our case, a
250 shortened washing step (only 5 min) is made possible by the quite high purity of the
251 crude sample (84 %) and the chemical nature of the impurities, which are all retained
252 by the exchanger. Only few impurities are eluted during this washing step (without
253 any displacer intervention). Experimental conditions are reported in Table 1. The
254 CPC chromatogram and HPLC fractogram that were obtained in these operating
255 conditions are shown in figure 4.

256

257 *Insert here figure 4*

258

259 The dirucotide purity was improved to 98 % (Table 1) without any chemical
260 degradation and critical impurities were removed by the IX-CPC process. However,
261 after injecting 200 mg of crude extract, a poor dirucotide recovery was obtained (48
262 %). The same operating conditions - DEHPA/dirucotide molar ratio = 100
263 (DEHPA/(positive charges of the analyte) = 25), a $[DEHPA]/[CaCl_2] = 10.4$, and
264 $[DEHPA]/[TEA] = 46.5$ - were applied for the purification of 402 mg and 1.0 g of
265 dirucotide crude extract.

266 As shown in Table 1, dirucotide purity was about 98 % in all runs and as expected,
267 the recovery was improved from 48 % to 69 % when increasing the injected mass
268 from 200 mg to 1 g.

269 The positive impact of mass overloading was confirmed by the HPLC fractogram of
270 the 400 mg and 1 g runs (see figure 5).

271

272 *Insert here figure 5*

273

274 The isotachic train organization was clearly improved when the injected sample mass
275 was increased while keeping the concentration constant, as usually observed in
276 displacement chromatography. [20,22] Indeed, the dirucotide emergence profile
277 became more and more rectangular-shaped while the compressive aspect of the
278 shock layers remained constant despite of the mass overloading, thus leading to an
279 increase of the pure dirucotide zone and therefore to a recovery improvement.

280 Another specificity of the displacement mode, observed on ~~the~~ figures 64 and 5, is
281 that the impurities accumulated in the shock layers because they were not
282 concentrated enough to form a complete rectangular eluting zone, as dirucotide did.
283 The resulting productivity was about 2.29 g per liter of stationary phase and per hour.
284 This productivity is indicative since the limited availability of the starting material did
285 not permit further study on the impact of the mass overload. Moreover, the mobile
286 phase consumption is of 180 L per kg of starting material, knowing that the mobile
287 phase consists of 90 % water. This consumption should be easily reduced by
288 increasing the amount of starting material injected per run.

289

290 **Conclusion**

291 The IX-CPC process, initially applied to dipeptide purification, succeeded to purify
292 dirucotide, a 17-aminoacid peptide, thus demonstrating its versatility. The high purity
293 and recovery (98 % and 69 % respectively) of purified dirucotide confirmed the high
294 selectivity of IX-CPC, since most of critical impurities were eliminated, even though
295 they are usually co-eluted with dirucotide in classical chromatographic separations.
296 Thus, IX-CPC revealed itself as an inexpensive, efficient and easy-to-implement
297 process for peptidic API polishing.

298

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304

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375 **CAPTIONS**

376 Figure 1: The chemical structure of dirucotide.

377 Figure 2: Schematic description of the IX-CPC process, DM: descending mode,

378 AMP: aqueous mobile phase, OSP: organic stationary phase, OUP: organic upper

379 phase, ALP: aqueous lower phase

380 Figure 3: HPLC chromatogram of crude synthetic dirucotide (main peak) showing the

381 main critical impurities (arrows) at $rt = 20, 20.5$ and 22.3 min, respectively impurities

382 C, A and B.

383 Figure 4. A- CPC chromatogram of the purification of 197 mg of dirucotide; B-

384 corresponding HPLC fractogram; C- HPLC analysis of dirucotide before and after

385 CPC purification.

386 Figure 5. HPLC fractograms of dirucotide purification by IX-CPC showing the positive

387 impact of mass-overloading (increase of the compressive aspect of the shock layers

388 and of the “length” of the purified dirucotide zone.

389 Table 1. Experimental conditions and experimental results

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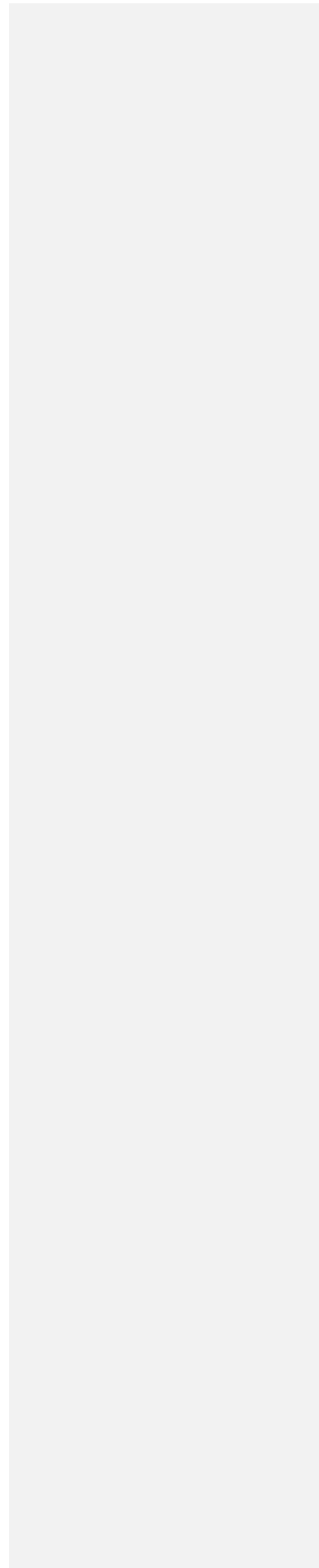
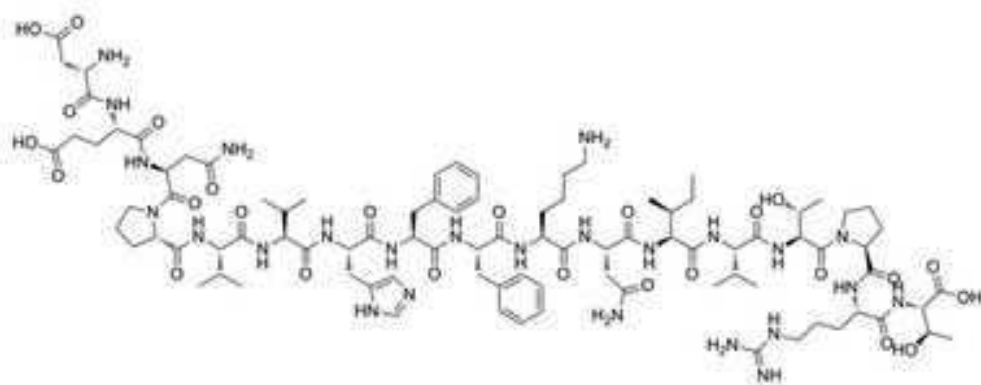


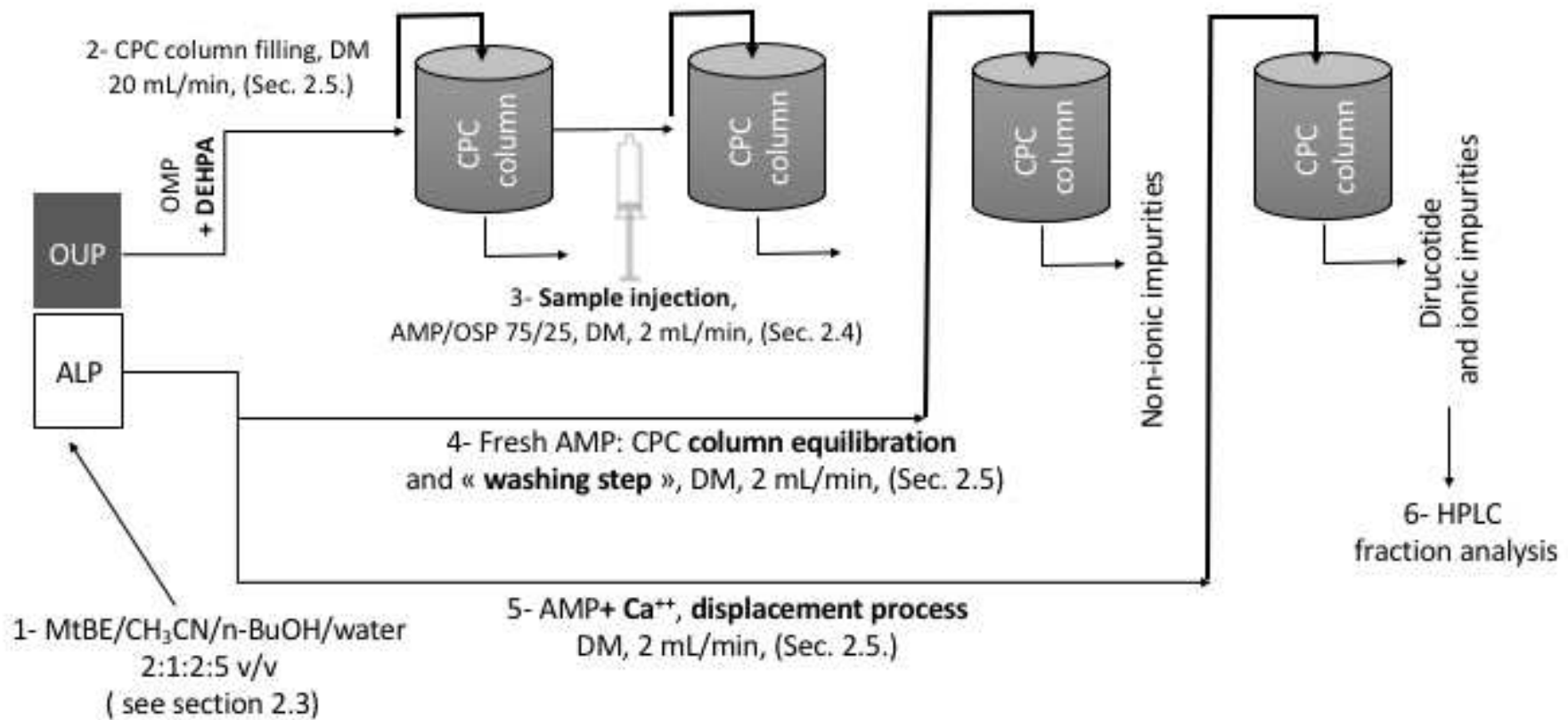
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Fig 1



Figure

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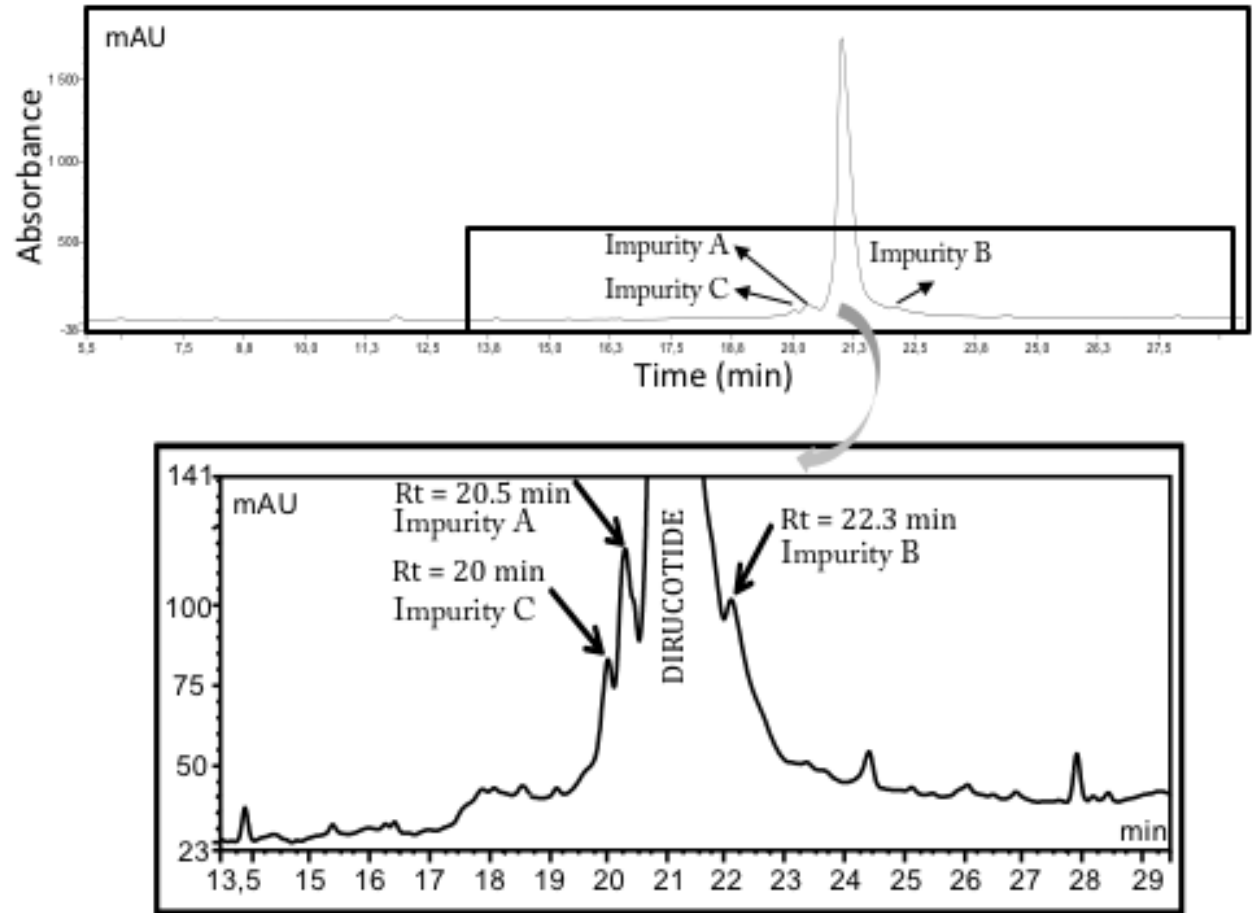


Fig. 4

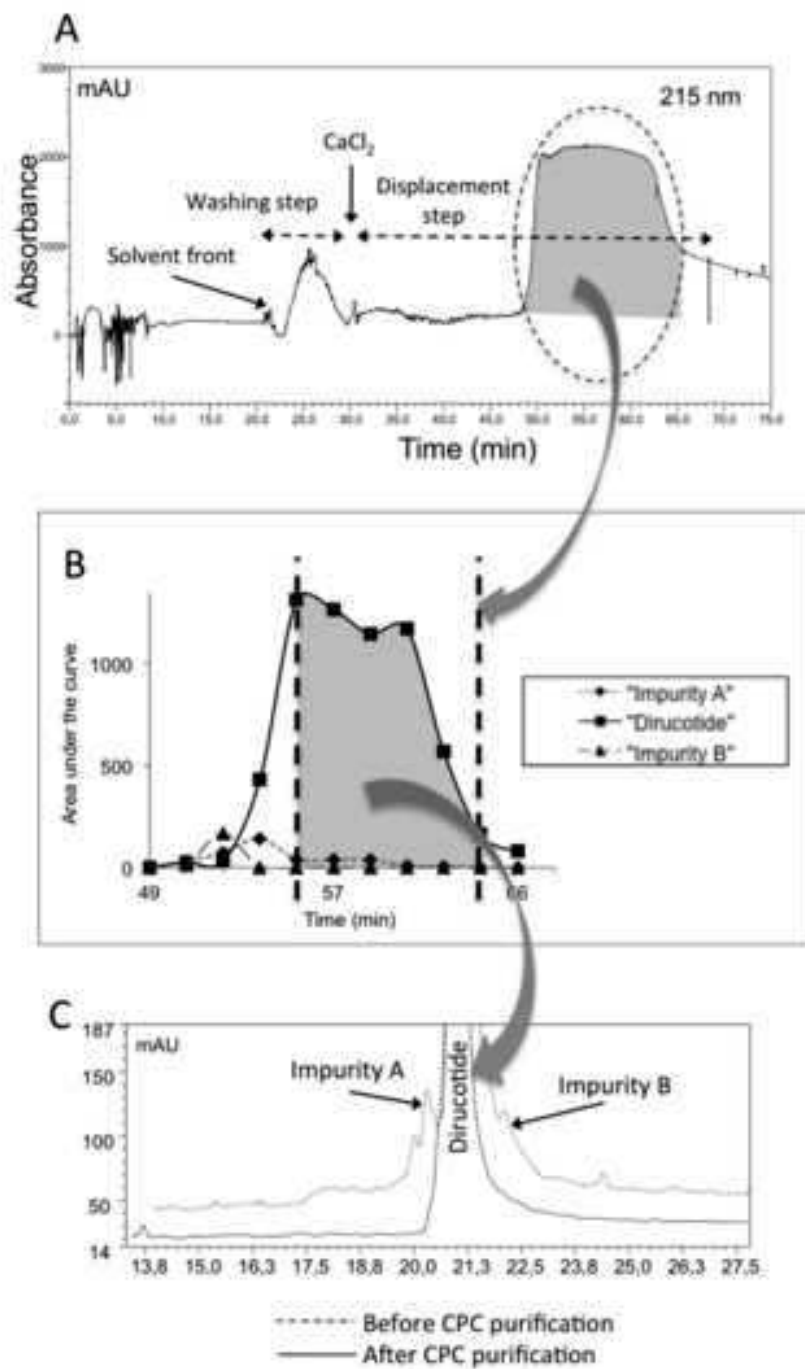


Figure
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Fig. 5

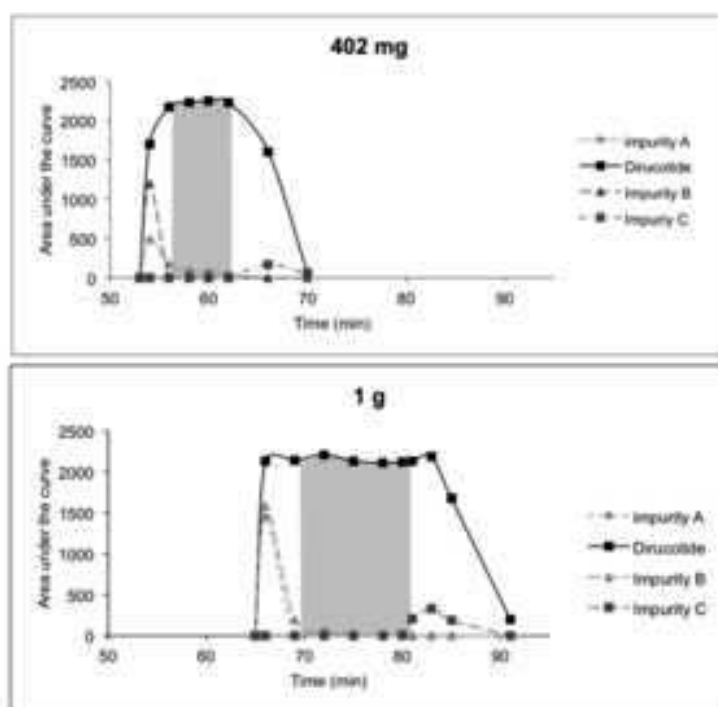


Table 1

EXPERIMENTAL CONDITIONS			
Injection mass (mg)	197	402	1000
Solvent system	MtBE/acetonitrile/ <i>n</i> -BuOH/water (2:1:2:5, v/v)		
Operating mode	Descending (aqueous phase as mobile phase)		
Exchanger (DEHPA) concentration (mM)	60	120	300
n_{DEHPA} /dirucotide	100	100	100
Displacer (CaCl ₂) concentration (mM)	5.76	11.5	28.8
Rotation speed (rpm)	1200		
Back pressure (bars)	35	35	30
Flow rate (mL.min ⁻¹)	2		
Stationary phase retention (%)	79	77	75
EXPERIMENTAL RESULTS			
Purified dirucotide (mg)	80	204.1	586.9
Dirucotide recovery (%)	48	60	69
Dirucotide purity (%)	98	98	98
Productivity (mg/h)	44	113.4	352.1