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Determination of digoxin in serum samples using a flow flow-through fluorosensor based on a molecularly imprinted polymer

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Abstract

This work describes the development of a competitive flow-through FIA assay for digoxin using a molecularly imprinted polymer (MIP) as the recognition phase. In previous work, a number of non-covalent imprinted polymers were synthesised by "bulk" polymerisation. The digoxin binding and elution characteristics of these MIPs were then evaluated to obtain a highly selective material for integration into a sensor. The optimum MIP was synthesised by photo-initiated polymerisation of a mixture containing digoxin, MAA, EDGMA and AIBN in acetonitrile. The bulk polymer was ground and sieved and the template removed by soxhlet extraction in MeOH/ACN. The MIP was packed into a flow cell and placed in a spectrofluorimeter to integrate the reaction and detection systems. The physical and chemical variables involved in digoxin determination by the sensor (nature and concentration of solution, flow rates, etc.) were optimised. Binding with the non-imprinted polymer (NIP) was also analysed. The new fluorosensor showed high selectivity and sensitivity, a detection limit of 1.7×10^{-2} µg L^{-1}, and high reproducibility (RSD of 1.03% and 1.77% for concentrations of 1.0×10^{-3} µg L^{-1} and 4.0×10^{-3} mg L^{-1} respectively). Selectivity was tested by determining the cross-

32 reactivity of several compounds with structures analogous to digoxin. Under the assay

33 conditions used, in which the potential interfering compounds were in concentrations

34 100 times higher than that of the analyte, no interference was recorded. The proposed

35 fluorosensor was successfully used to determine digoxin concentration of human serum

36 samples.

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38 *Keywords:* Digoxin; Molecular imprinting; Fluorosensor; Human serum analysis

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40 **1. Introduction**

41   The use of molecular imprinting in the design of new drug delivery systems and

42 devices has attracted much attention in recent years. Molecularly imprinted polymers

43 (MIPs) combine highly selective molecular recognition properties (comparable to those

44 of biological systems) with characteristics such as physical robustness and good

45 thermal, chemical and mechanical stability. This renders them particularly suitable for

46 use as recognition elements in sensor technology (D'Agostino et al., 2006; Huang et al.,

47 2007). Further, these materials can be employed in aqueous and non-aqueous media and

48 can be manufactured in different configurations (e.g., as blocks, beads, microspheres,

49 thin-films, filaments or microstructures) to facilitate their integration into sensor design

50 (Lakshmi et al., 2006; Breton et al., 2006). MIPs can also be used in chromatographic

51 separation (Watabe et. al., 2005), as selective adsorbents for cleaning samples (Chapuis

52 et. al., 2006), in solid-phase extraction (Hu et. al., 2005; Baggiani et. al., 2007), and as

53 catalysts (Vokmann and Brüggermann, 2006). Molecular imprinting is now an

54 established technique for the production of molecular recognition materials with

55 predetermined affinities for analytes such as amino acids (Li and Husson, 2006),

56 proteins (Bossi et. al., 2007), carbohydrates (Furqan and Hansen, 2007), drug

57   compounds (Suede et. al., 2006), pesticides (Wei et. al., 2006), steroids, corticosteroids

58   (Sun et. al., 2006), and metal ions (Kidschy and Alocilja, 2005).

59   Digoxin is a glycoside used in the treatment of congestive heart failure; in fact it

60   has been used for this for over 200 years and is still one of the most widely prescribed

61   heart failure drugs. Strict control of digoxin therapy is necessary, however, since there is

62   a thin line separating therapeutic and toxic levels (0.05-0.2 μg l^{-1}); sensitive and

63   selective detection techniques are therefore required. This paper describes a flow-

64   injection optical sensor for digoxin that combines sensor technology with a new

65   generation of molecularly imprinted synthetic receptors. Preliminary work on the

66   composition and synthesis conditions of this MIP has been reported (Paniagua et. al.,

67   2006); this allowed the most suitable MIP for the new fluorosensor for digoxin to be

68   chosen. MIPs were synthesised under different conditions, i.e., changing the functional

69   monomer employed (methacrylic acid or 2-vynilpyridine) as well as the porogen

70   (acetonitrile or dichloromethane). The polymerisation process was studied under UV

71   light (365 nm) or in a thermostat-controlled waterbath (60ºC) for 8 to 14 h. The binding

72   and elution solutions, the concentration of the labelled antigen solution and the flow rate

73   were all optimised. The binding of digoxin to the non-imprinted polymer (NIP) was

74   also examined under optimum conditions. The proposed fluorosensor was highly

75   selective and sensitive and provided highly reproducible results. The proposed

76   fluorosensor was successfully used to determine the digoxin concentration of human

77   serum samples.

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2. Material and Methods

2.1. Reagents

Ethylene glycol dimethacrylate (EDMA) and methacrylic acid (MAA) were purchased from Merck (Darmstadt, Germany), 2-2′-azobisisobutyronitrile (AIBN) from Fluka (Buchs, Switzerland), and digoxin (95%), morphine, heroine, codeine, tebaine, pentazocine and narcotine from Sigma Aldrich (Madrid, Spain). Digoxin labelled with fluorescein isothiocyanate (FITC) (10 μmol l^{-1}) was obtained from MicroPharm (Newcastle, Carmarthenshire, UK). Acetonitrile (ACN) and methanol (HPLC grade) were supplied by Scharlau (Barcelona, Spain). Phosphate buffer solution (PBS, pH=7.5) (NaCl 0.1 mM; KH_2PO_4 1.4 mM; KCl 2.7 mM; NaH_2PO_4 8 mM; $MgCl_2$ 21.3 mM), anhydrous sodium carbonate and sodium dodecylsulphate (SDS) were obtained from Merck (Darmstadt, Germany). Deionised water (18.3 MΩ cm) used in the preparation of aqueous solutions was obtained using a Milli-Q water system (Millipore Ibérica, Madrid, Spain).

2.2. Instrumentation

Fluorescence intensity was measured with a Perkin-Elmer LS-5 spectrofluorimeter equipped with a 100 μl Hellma (Jamaica, NY, USA) flow cell (optical path 3 mm) in conjunction with an AAT computer. The flow-injection system consisted of a Gilson Minipulse-2 peristaltic pump and an Omnifit six-way injection valve. PTF tubes (0.5 mm i.d) were used to build the manifold. The pH was measured using a Metrohm 654 pH meter. An ultraviolet lamp (Vilber Lourmat CN-6T) was used to induce polymerisation. The morphology of the polymer was characterised using a Jeol JSM-6400 scanning electron microscope (SEM). The surface area of the imprinted

106 polymer was characterized using a Micromeritics ASAP 2000 apparatus (Norcross,

107 USA).

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109 *2.3. Procedures*

110 *Preparation of the molecularly imprinted polymer*

111   In preliminary work (Paniagua et. al., 2006), a number of polymers were

112 prepared by bulk polymerisation under different synthesis conditions in order to select

113 that with the best digoxin-recognition characteristics. Several functional monomers

114 (methacrylic acid or 2-vinylpyridine) and different types of porogen (ACN, chloroform

115 or dichloromethane) were tested. The best polymerisation procedure (either employing

116 a UV source or a thermostat-controlled waterbath) and extraction process (soxhlet or

117 microwave extraction) were also determined. The surface morphology of the polymers

118 was analysed by SEM, and the binding affinity of the different digoxin-MIPs evaluated

119 by equilibrium binding experiments. The molecular ratio of the optimum polymer was

120 $(10^{-3}:1:5)$ digoxin:MAA:EDMA. The fluorosensor was equipped with this polymer,

121 which was prepared in a glass tube by the bulk polymerisation method using a mixture

122 of the template molecule $(2.0\times10^{-3}$ mmol), methacrylic acid (2.0 mmol), ethylene glycol

123 dimethacrylate (10.0 mmol), 2-2´-azobisisobutyronitrile $(6.0\times10^{-2}$ mmol), and 10 ml of

124 ACN as a porogen. The pre-polymerisation mixture was degassed with nitrogen for 5-

125 10 min. A control polymer were prepared using the same composition but in the

126 absence of the template. The glass tube was then exposed to the UV source (λ/nm 365

127 nm) at 10ºC for 24 h. After polymerisation, the polymer block was removed from the

128 glass tube and was manually ground in a mortar and sieved to a particle size of 355-600

129 μm. The template was extracted by the Soxhlet system with MeOH:ACN (50:50, v/v)

130 over a period of 20 h.

131   *Preparation of the fluorosensor*

132   Figure 1 shows the fluorosensor apparatus. The reactor was a flow-through cell

133   (100 μl) packed with the sensitive phase (digoxin-MIP); this was placed in the

134   spectrofluorimeter, thus integrating the reaction and detection systems. The flow stream

135   was generated by an upstream peristaltic pump. The samples were introduced into the

136   system by a six-way valve equipped with a 150 μl sample loop.

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138   *Sample preparation*

139   Digoxin-containing serum samples were supplied by the Puerta de Hierro

140   Hospital (Madrid). These were stored at 4ºC. Sample aliquots of 650 μl were added to

141   900 μl of ACN and centrifuged at 3500 rpm for 30 min to precipitate the proteins.

142   *Competitive assay protocol for the determination of digoxin in serum samples*

143   To determine the digoxin concentration of the serum samples, a heterogeneous

144   fluorescent competitive assay was undertaken in which the digoxin competed with a

145   fluorescent tracer (FITC-digoxin) for recognition sites in the digoxin-MIP. For this,

146   1700 μl of purified serum was mixed with 170 μl of FITC-digoxin (0.2 μmol l^{-1}). The

147   competitive calibration curve was obtained using digoxin solutions (1000 μl) at

148   different concentrations (0-4×10^{-3} mg l^{-1}) in ACN. This required a digoxin stock

149   solution (0.5 ppm) be prepared in a mixture of ACN and Na_2CO_3 (0.1 M, pH=8.0)

150   (50:50 v/v). For the preparation of the standard solutions, different aliquots of the 0.5

151   stock solution were added to 100 μl of FITC-digoxin (1:50 in ACN, 0.2 μmol l^{-1}), and

152   ACN then added to 1000 μl. 150 μl of corresponding standard were then injected into

153   the carrier solution (ACN) at a flow rate of 0.27 ml min^{-1}. Excess antigens, labelled and

154   unlabelled, were removed by the carrier solution. The fluorescence signal generated was

155 measured *in situ* in the reactor at λ_{em}/nm 517 and λ_{exc}/nm 496. Finally, an elution

156 solution (MeOH/ACN, 90:10) was pumped into the flow cell to regenerate the reactor.

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158 **3. Results and Discussion**

159 *3.1. Optimisation of experimental conditions and characterisation of the sensor*

160 The sensitivity, selectivity and response time of the fluorescent sensor were

161 determined. The composition of the polymer and its polymerisation conditions were

162 determined in previous work (Paniagua et. al., 2006). The greatest specific binding was

163 achieved with ACN (42%). This solvent showed a very low binding affinity for the

164 control polymer; non-specific binding was 19%. The percentage uptake with the

165 imprinted polymer was 61%. Using this information it was possible to select the

166 polymer best suited to act as the recognition phase in the proposed sensor.

167 The effect of polymerisation on the particle structure of the methacrylic polymer

168 was also examined. The surface area, specific pore volume and average pore diameter

169 of the polymer were obtained by nitrogen sorption porosimetry. The MIP had a specific

170 surface area of 31.844 m^2 g^{-1} and a specific pore volume of 0.194 cm^3 g^{-1}. The pore size

171 distribution was macropores 50%, mesopores 44.33%, and micropores 5.67%.

172 The study of the binding mechanism and regeneration of the polymer was

173 carried out. The efficacy of the retention process was affected by the carrier solvent, pH,

174 and the flow rate. The amount of labelled digoxin used was optimised in order to obtain

175 good sensitivity and a strong fluorescence signal. For this, 150 µl of digoxin-FITC at

176 different concentrations (0.0075; 0.15; 0.2; 0.3 µmol l^{-1}) plus several carrier solvents

177 (ACN, MeOH and aqueous phosphate buffer solution PBS, pH=7.5) were assayed by

178 spectrofluorimetry at λ_{exc}/ nm 496 (λ_{em}/nm 517). Figure 2 shows the fluorescence

179 signals obtained using different binding solutions for different concentrations of FITC-

180   digoxin. The effect of the binding solution flow rate was studied in the range 0.1 to 0.6

181   ml min^{-1}. The effect of pH (3-9) was also tested. ACN at pH 9.0 was finally selected as

182   the carrier solution. The optimum flow rate was 0.27 mL min^{-1}. With a slightly higher

183   flow rate (0.3 ml min^{-1}) no improvements were seen; higher flow rates considerably

184   reduced the retention of the polymer. To increase the strength of the fluorescence

185   signals, the tensative effect of different concentrations (4.0, 6.0, 8.0 and 10.0 mM) of

186   SDS was examined; 100 µl SDS of each concentration were added to 50 ml of ACN.

187   The best fluorosensor response was obtained when working close to the critical micellar

188   concentration (8.1 mM) of SDS; the optimum value was 8.0 mM.

189   To prevent the polymer losing binding capacity, the optimum washing time was

190   determined, and regeneration solutions used to regenerate the reactive phase. The effect

191   of a 30-120 s washing time was studied for a 0.2 µmol l^{-1} digoxin-FITC solution; the

192   optimum time was 80 s. Shorter times did not allow the polymer to regenerate, thus

193   reducing its binding capacity. Longer times did not improve the process. MeOH:H$_2$O

194   (70:30, 50:50 and 30:70), MeOH:ACN (90:10), MeOH:ACN:H$_2$O (80:10:10) and

195   MeOH and H$_2$O were all tested as regeneration solutions. Figure 3 shows their elution.

196   MeOH:ACN (90:10) provided the best results.

197   A binding assay using NIP as the reactive phase was undertaken under optimum

198   conditions. No digoxin bound to the NIP.

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200   *3.2. Analytical performance*

201   To determinate the selectivity of the MIP, cross-reactivity with narcotine,

202   tebaine, heroine, pentazocine, morphine and codeine was examined. For interference

203   studies, competitive calibration curves were plotted, using concentrations of 0-6×10^2 µg

204   l^{-1} of digoxin. The ratios between the I$_{50}$ values determined for digoxin and the

205 potentially interfering substances were calculated. In no case was cross reactivity

206 detected, even when the concentration of the test molecules was 100 times that of the

207 analyte (Fig. 5).

208   A competitive calibration curve was obtained for the working range of $0-4\times10^{-3}$

209 mg l^{-1} digoxin and 0.2 µmol l^{-1} of FITC-digoxin. The normalized fluorescence signal,

210 %B/Bo (where B is the intensity of fluorescence of the conjugated digoxin at different

211 standard concentrations of digoxin, and Bo that of the blank), was plotted against the

212 digoxin concentration. Figure 4 shows the calibration curve obtained. The curve

213 equation was: %B/Bo= $9\times10^{6}\,C^{2} - 5\times10^{4}\,C + 96$ (n=5, r=0.990). The detection limit (X-

214 2SD) was calculated using five replicates of zero standards and expressed as the least

215 detectable dose (LDD) of digoxin; under optimum conditions this was 1.7×10^{-2} µg l^{-1}.

216 The relative standard deviations (RSD) of six determinations for 1×10^{-3} and 4×10^{-3} mgl^{-1}

217 digoxin were 1.0% and 1.8% respectively; for the serum samples an RSD of <10% was

218 obtained. The total time required for each assay was 400 s. The lifetime of the sensor

219 without loss of sensitivity was approximately 18 months; no special storage conditions

220 were necessary to maintain optimum performance.

221

222 *3.3. Sample analysis application*

223   Table 1 shows the results of the determination of digoxin in the serum samples

224 obtained using the proposed method and a reference method (MEIA, microparticle

225 immunoenzyme assay, Abbott Laboratories). No significant differences were seen

226 between the values obtained ($p<0.05$).

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230 **4. Conclusions**

231   This paper proposes a new molecularly imprinted fluorescence sensor for
232 determining the concentration of digoxin in serum. Strategies for selecting the best
233 combination of monomers, cross-linkers, solvents and polymerisation conditions for
234 production of the MIP were established. This included determining the best morphology
235 of the MIP and the optimum pore size. The polymer finally chosen showed good
236 digoxin-recognition properties and was sufficiently stable for integration into a flow-
237 through fluorosensor. The imprinted polymer showed good thermal (up to 80ºC) and
238 chemical stabilities and can be used over the pH range 3-9; no digoxin bound to the
239 NIP. Cross-reactivity with compounds of similar structures was negligible.
240 Consequently, this fluorosensor is highly sensitive and selective for digoxin. The
241 analysis and regeneration time is very short (about 400 s). When used to determine
242 digoxin concentrations in human serum, the fluorosensor provided satisfactory results.

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249   **References**

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278 **FIGURE LEGENDS**

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280   Fig. 1. The flow-through sensor system.

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282   Fig. 2. Binding studies on MIP for different concentrations of digoxin-FITC and

283   solvents. Carrier solutions: ACN, MeOH, PBS (10 mM, pH= 7.5). Flow rate: 0.27 ml

284   min^{-1}. Digoxin-FITC concentrations (7.5×10^{-3}; 0.15; 0.2; 0.3 µmol l^{-1}).

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286   Fig .3. Elution percentages for different solutions. Carrier solution: ACN (pH= 9; SDS

287   8mM). Flow rate: 0.27 ml min^{-1}, [digoxin-FITC]= 0.2 µmol l^{-1}.

288

289   Fig. 4. Calibration curve for the optimum sensor, obtained by plotting the normalized

290   signal against the digoxin concentration.

291

292   Fig. 5. Calibration curves obtained with the optimum fluorosensor for digoxin and

293   structurally analogous compounds.

294

295 **TABLES**

296 Table 1

297 Results of human serum samples analysis

Sample	Fluorosensor $10^{-4}\,(X \pm SD)/ng\,\mu l^{-1}$	Reference method $10^{-4}\,(X \pm SD)/ng\,\mu l^{-1}$
1	(7.6 ± 0.8)	(8.0 ± 0.3)
2	(2.5 ± 0.2)	(3.0 ± 0.2)
3	(21.0 ± 2.1)	(24.2 ± 1.3)
4	(9.1 ± 0.6)	(9.0 ± 0.3)
5	(15.2 ± 0.9)	(19.0 ± 2.1)
6	(8.4 ± 0.7)	(8.0 ± 0.4)

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299

300   **FIGURES:**

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307   Spectrofluorimeter   Computer

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310   Inyection valve
                                            (six ways)

311   ACN
                                                     (carrier solution)

312   PERKIN ELMER
        LS-5
        SPECTROFLUORIMETER

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315   Waste

316   Hypodermic syringe   MeOH/ACN

317   Flow cell   Peristaltic pump

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320   Fig.1

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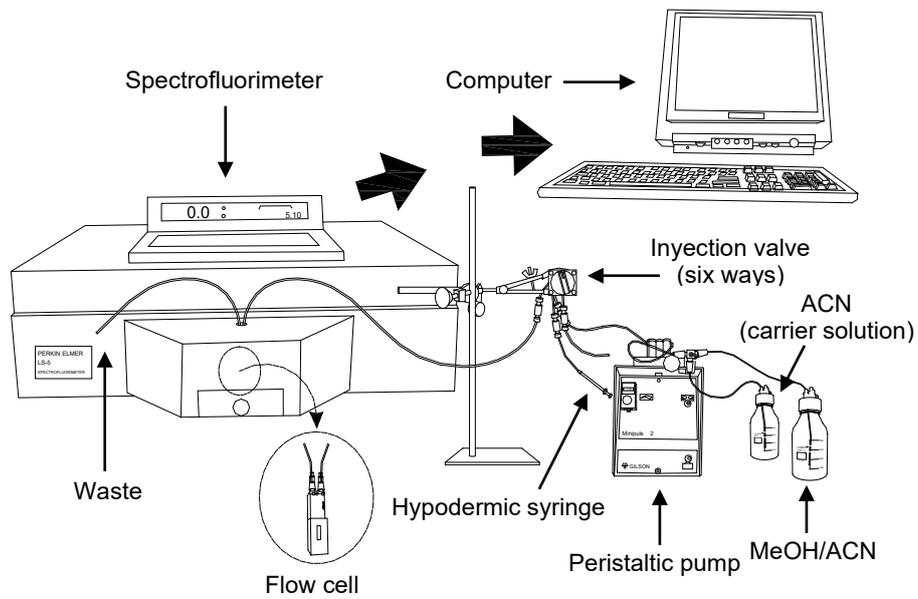
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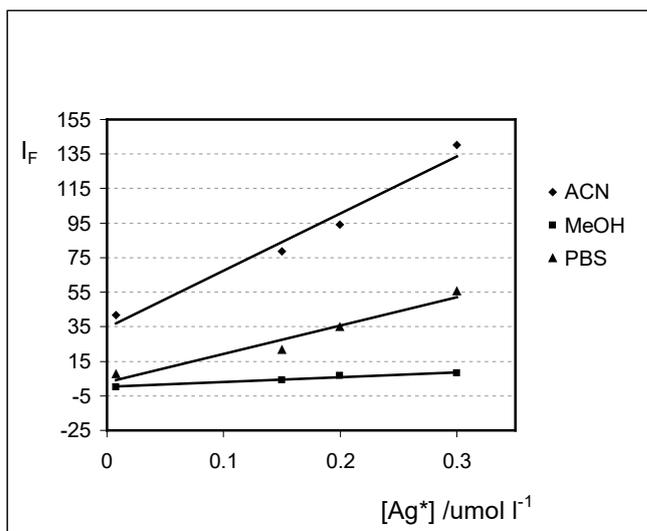
Fig.2

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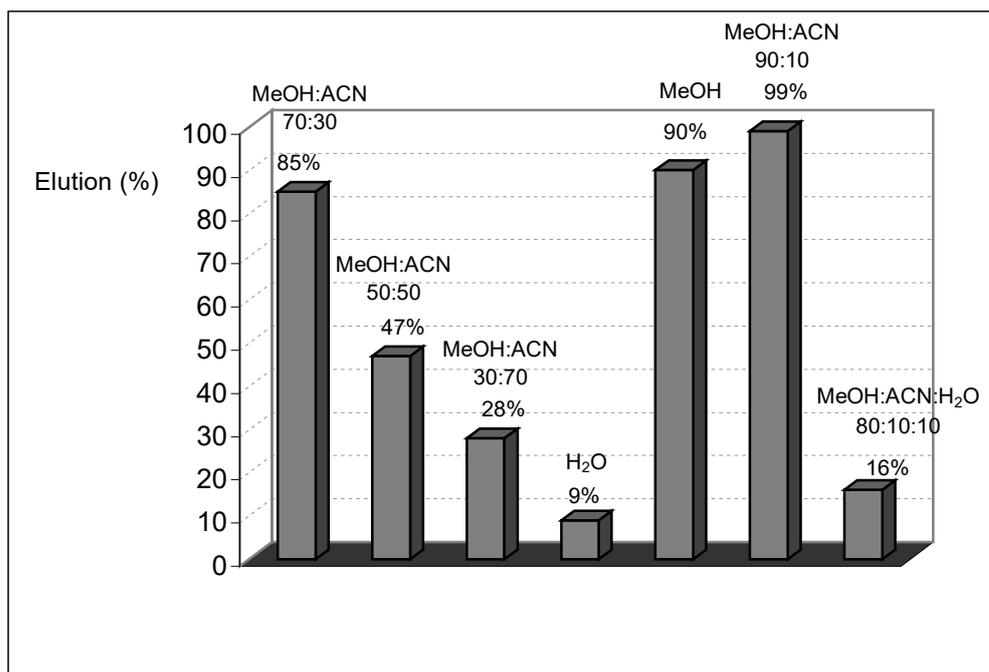
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343   Fig.3

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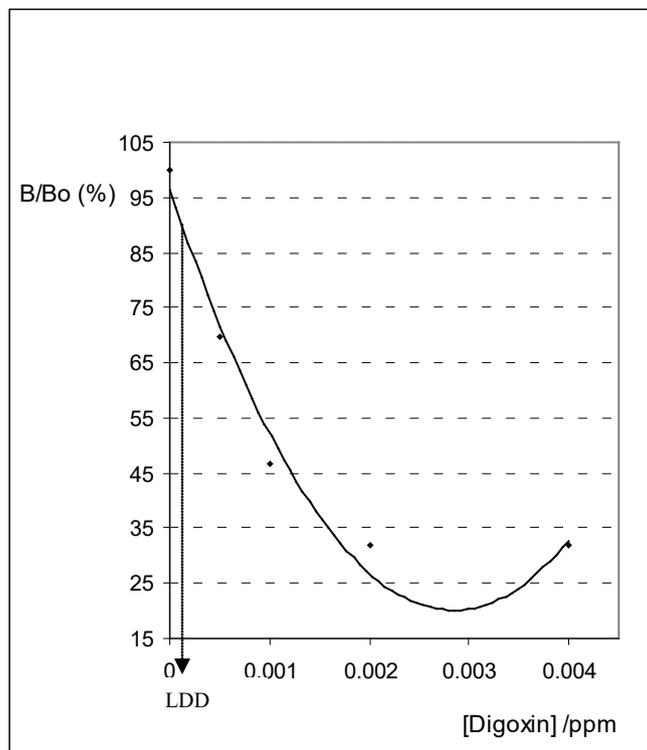
Fig.4
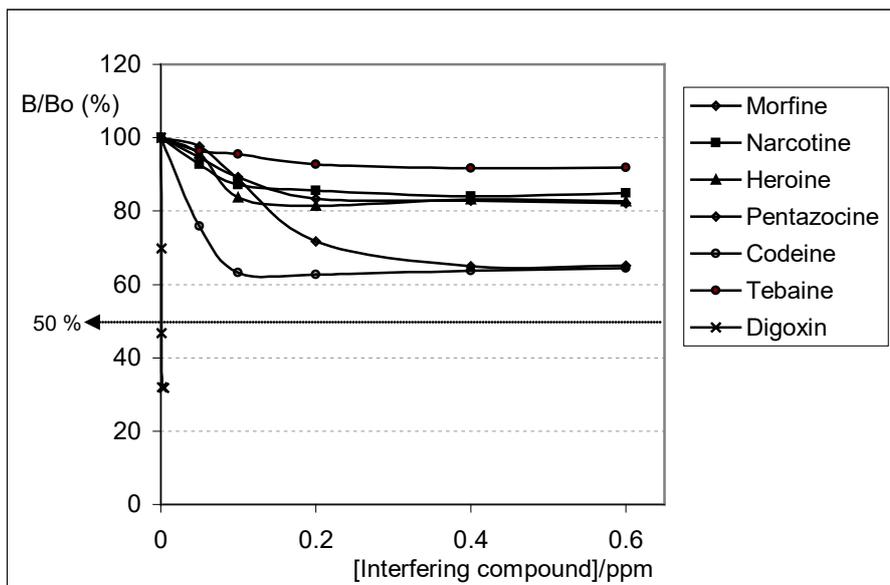
Fig. 5

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