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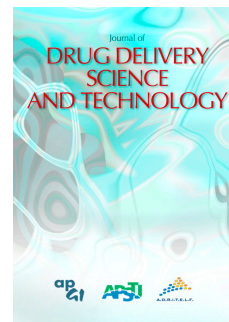
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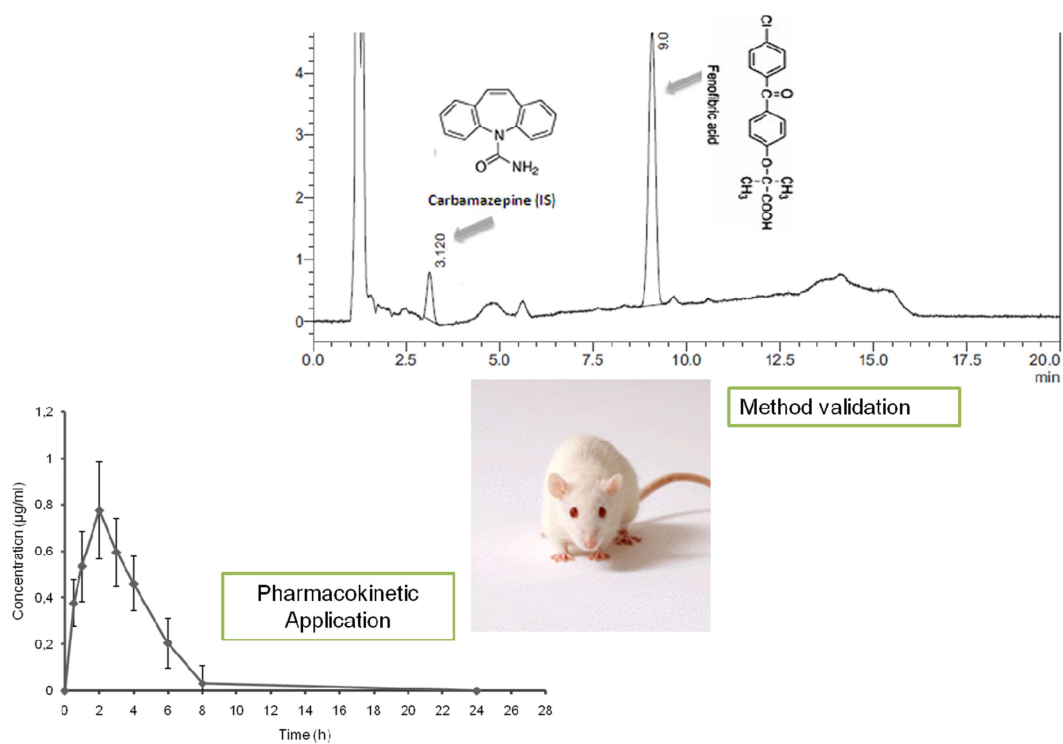
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1 **Use of mouse model in pharmacokinetics study of poorly**
2 **water soluble drugs: Application to fenofibrate**

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18 **ABSTRACT**

19

20 Fenofibrate has recently been used as drug model in several studies with the objective of
21 optimizing the development of some drug delivery systems to overcome the problem of poor
22 aqueous solubility of the newly discovered API. The adequacy of the drug delivery systems to
23 improve the oral bioavailability of encapsulated drug is generally evaluated by a
24 pharmacokinetic study. The use of mouse as animal model for pharmacokinetic studies has
25 become more important in the last decade because of many similarities with the human model
26 in terms of the mechanisms of absorption, metabolism and elimination. Nevertheless, the
27 mouse is often hampered by the very small volumes of blood that could be obtained during
28 sampling. The aim of this work was to overcome the problem of lower volumes of plasma
29 withdrawn by developing an appropriate protocol for sample preparation and a suitable HPLC
30 method for drug quantification in mouse plasma. Linear calibration curve was obtained over the
31 concentration range from 0,16µg/mL to 32µg/mL ($r^2=0,9999$) with LLOQ of 0,16µg/mL The
32 RSD in both intra-run and inter-run precision study was less than 11% and the extraction
33 recoveries were above 91.9%. The reproducible method was successfully applied to the
34 pharmacokinetic study of fenofibrate in mouse.

35

36 **KEY WORDS:** Fenofibric acid, HPLC-UV, Pharmacokinetic, mouse, fenofibrate, poorly
37 water soluble drugs

38 INTRODUCTION

39 Fenofibrate, is a well-studied lipid regulating agent used for the treatment of
40 hypercholesterolemia and hyper-triglyceridemia. After absorption, the drug is completely
41 hydrolyzed by esterases in its active metabolite, the fenofibric acid, [1] which is eliminated
42 with a half-life of 20 hours in human after one daily dosage. Its maximal plasma concentration
43 is obtained around 3 to 4 hours post dosage.

44 Fenofibrate is practically insoluble in water and exhibits an extremely poor oral
45 bioavailability [2]. Therefore, fenofibrate has recently been used as drug model in several
46 studies with the objective of optimizing the development of some drug delivery systems to
47 overcome the problem of poor aqueous solubility characterizing more than 50% of the newly
48 discovered API. Among these systems : solid lipid nanoparticles [3] nanocrystals [4],
49 nanosuspension [5], selfmicroemulsifying systems [6] that were developed using fenofibrate as
50 a drug model . The adequacy of these drug delivery systems to improve the oral bioavailability
51 of encapsulated drug is generally evaluated by a pharmacokinetic study which requires a
52 suitable animal species that should closely resembles to human in terms of the mechanisms of
53 absorption and elimination of the active ingredient.

54 The rat appears to be the primary species most commonly used in preclinical
55 pharmacokinetic studies. However, it was shown that the oral bioavailability values and the
56 metabolic enzymatic levels in intestine are distinct between humans and rats [7].

57 Moreover, the use of the mouse as an animal model for pharmacokinetic studies has become
58 more important in predicting oral bioavailability due to several similarities in the absorption,
59 metabolism and elimination processes between humans and mice [8]. Besides, the presence of gall
60 bladder in mice led to the occurrence of an absorption mechanism, entitled collisional transfer,
61 by which highly lipophilic drugs are diffused through the glycocalyx when they are intercalated
62 in the bile acids (fasting state) or in the mixed micelles of bile acids (fasted state) [9]. As rats
63 do not possess a gall bladder, we chose to perform our study in mice. Nevertheless, the mouse
64 exhibits a particular challenge because of the very small volumes of blood that could be
65 obtained during sampling and hence impediments for pharmacokinetics [8, 10].

66
67 The aim of this work was at first to overcome the problem of lower volumes of plasma
68 withdrawn by developing a one-step processing method for samples preparation that would be
69 rapid in applying and ensuring at least 90% of drug extraction efficiency. Secondly, a fast and

70 sensitive analytical method was developed for the dosage of the active metabolite of fenofibrate
71 (fenofibric acid) by HPLC-UV.

72
73 This quantification method was subsequently validated according to the recommendations of
74 the SFSTP [11] and the FDA guideline for validation of bioanalytical methods[12]. The
75 feasibility of the animal model and the extraction protocol were evaluated by carrying out a
76 pharmacokinetic application to fenofibrate in its commercialized form LIPANTHYL® with an
77 oral dose corresponding to 5 mg / kg.

78

79

80 **2. MATERIALS AND METHODS**

81 *2.1. Chemicals and reagents*

82 Fenofibric acid (purity $\geq 98.0\%$) was provided from Sigma-Aldrich and the internal
83 standard (I.S.), carbamazepine ($\geq 99.0\%$ purity) was purchased from Sigma-Aldrich (China).
84 Methanol and deionized water of HPLC grade were bought from Carlo Erba (France).

85 Ammonium acetate was of analytical grade was purchased from Sigma-Aldrich (Netherlands)
86 Hydrochloric acid and diethyl ether were acquired from Carlo Erba (France).. Normal mouse
87 plasma was brought from Sigma-Aldrich (USA) and stored at -20°C .

88

89 *2.2. Chromatographic conditions*

90 Analyses were performed on a Shimadzu HPLC Class VP series (Shimadzu, Kyoto,
91 Japan) which was composed of a LC-10ADvp quadratic pump, an auto-sampler (model SIL-
92 10Avp Shimadzu), a variable UV-Visible wavelength detector (model SPD 10Avp; Shimadzu)
93 and the data were analyzed by Class-VP series software, version 5.03 (Shimadzu, Japan). The
94 analytical column used was LiChrospher 100 RP8 (125mm \times 4.6mm I.D, 5 μm particle size). The
95 detection wavelength was fixed at 287 nm and the column oven temperature was set at
96 35 $^{\circ}\text{C}$. Elution was obtained by applying the gradient steps summarized in table 1 and
97 corresponding to solvents A (Methanol) and B ((5 mM ammonium acetate buffer (pH 3.3)). In
98 all cases the flow rate was 1.5 ml/mn and the UV detection was achieved at a wavelength of 287
99 nm.

100 **Table 1.** Gradient elution system for separation of fenofibric acid and internal standard

Cumulative time of acquisition (min)	Gradient	
	% Solvent A Methanol	% Solvent B 5 mM ammonium acetate buffer (pH 3.3)
5	56	44
10.5	66	34
12.5	76	24
13	66	34

101

102 **2.3. Preparation of standard and stock solutions:**

103 The Stock solution of fenofibric acid was prepared in methanol at a concentration of
104 1600 μ g/ml.

105 In the other hand, the preparation of the internal standard stock solution was done at a
106 concentration of 4.7 μ g/ml by dissolving 2.35 mg carbamazepine in 500 ml HCl (1M).

107 Then, the standard solutions of fenofibric acid with concentrations of 1600, 800, 500, 160, 80,
108 16 and 8 μ g/ml were prepared after serial volumetric dilution of fenofibric acid stock solution
109 in methanol.

110 These solutions were prepared and stored in appropriate conditions (at -20°C) until analysis.

111

112 **2.2. Extraction methods and sample preparation procedure:**

113 25 μ L aliquot of mouse plasma was mixed with 12.5 μ L of the internal standard solution in
114 1.5ml Eppendorff tube.

115 Precipitation of the plasma proteins has been carried out by adding 250 μ L of methanol then
116 by vortexing the mixture for 30 minutes using a vortex mixer (Eppendorf, Hamburg,
117 Germany).. Afterward the samples were placed in an ice bath for 60 min, followed by
118 centrifugation for 10 minutes at 12.000 rpm (4 ° C). The top layer was injected (20 μ L) into
119 the HPLC system for analysis.

120

121 **2.4. Calibration curves and quality control plasma samples preparation**

122 Calibration curves of fenofibric acid were prepared with concentration levels of 32,
123 16, 3.2, 1.6, 0.32 and 0.16 $\mu\text{g/ml}$ by spiking 0.5 μL of the standard solution in 24.5 μL of
124 mouse plasma. This ratio of 2:100 was chosen in order to avoid plasma alteration with the
125 standard solution [13].

126 Three control samples (QC) were prepared in blank mouse plasma at different concentrations
127 of fenofibric acid (0.64, 16 and 25.6) $\mu\text{g/ml}$. All samples were conserved at $-80\text{ }^{\circ}\text{C}$ until
128 analysis.

129 A single set of quality controls and standards were analyzed each day of the overall work
130 using the same procedure described above for plasma samples.

131

132 **2.6 Method validation:**

133 A complete validation of the used method for the determination of fenofibric acid in
134 mouse plasma was done according to the FDA guideline for validation of bioanalytical
135 methods [12] and to the SFSTP recommendations [11].

136 The method has been validated for selectivity, linearity, lower limit of quantification (LLOQ),
137 precision and accuracy, stability and recovery.

138 **2.6.1. Selectivity**

139 Chromatograms of drug-free mouse plasma were compared ($n=6$). All samples were
140 processed using the protein precipitation procedure in order to ensure the absence of
141 endogenous peaks co-eluted with fenofibric acid or the internal standard.

142 **2.6.2. Precision and accuracy**

143 To assess the intra- and inter-day accuracy and precision, repetitive measurements of
144 the three QC sample concentrations (0.64, 16 and 25.6 $\mu\text{g/ml}$) were realized. Intra-day
145 accuracy was assessed through the QC samples analyzed in replicates of five per day. The
146 inter-day precision was determined by performing the same procedure once a day during three
147 consecutive days. Precision and accuracy were expressed as relative standard deviation (RSD)
148 and relative error (RE) respectively. The precision and accuracy acceptance criterion for each
149 QC sample concentration was within $\pm 15\%$.

150 **2.6.3. Linearity and limit of quantification**

151 To assess the linearity, six-point standard curves had been run within the concentration
152 range 0.16 - 32 μ g/ml on three different days with replicates of four for each concentration per
153 day (n=4).

154 Standard curves were fitted by plotting peak area ratio of fenofibric acid to internal standard
155 versus the corresponding concentration. Calibration curves were obtained by least-squares
156 linear regression method using weight scheme as x (x= concentration)

157 The acceptance criterion of the coefficient of correlation for the calibration curves was
158 0.999 or greater and every back-determined standard concentration had to be within 15%
159 deviation with the exception of the lower limit of quantification (LLOQ) that was within 20%.
160 The LLOQ is corresponding to the lowest concentration on the standard curve which could be
161 evaluated with adequate precision and accuracy.

162 **2.6.4. Recovery**

163 Extraction recovery was calculated by analyzing the three QC samples (three samples
164 each) in order to evaluate the extraction performance of the used method.

165 The recoveries had been evaluated by comparing the peak areas of the extracted samples with
166 those in which the analytes were subsequently added.

167 **2.6.5. Stability study**

168 Stability of fenofibric acid in mouse plasma during processing and storage was
169 checked using the QC samples at three distinct concentrations that were analyzed under
170 different conditions.

171 QC samples were analyzed after short-term storing at room temperature for 24 h, after long-
172 term storage : one month at -20°C, and after three freeze-thaw stability cycles. The post-
173 processing stability was also evaluated by analyzing reconstituted QC samples that were kept
174 in auto-sampler for 24 h.

175 Every stability study was carried out in replicates of four for each QC sample..

176 **2.7. Application to pharmacokinetic study**

177 The *in vivo* study was carried out according to the EU Directive 2010/63/EU related
178 to animal experiments. 8-week-old male BALB/cJRj mice (Janvier, St. Genest de Lisle,

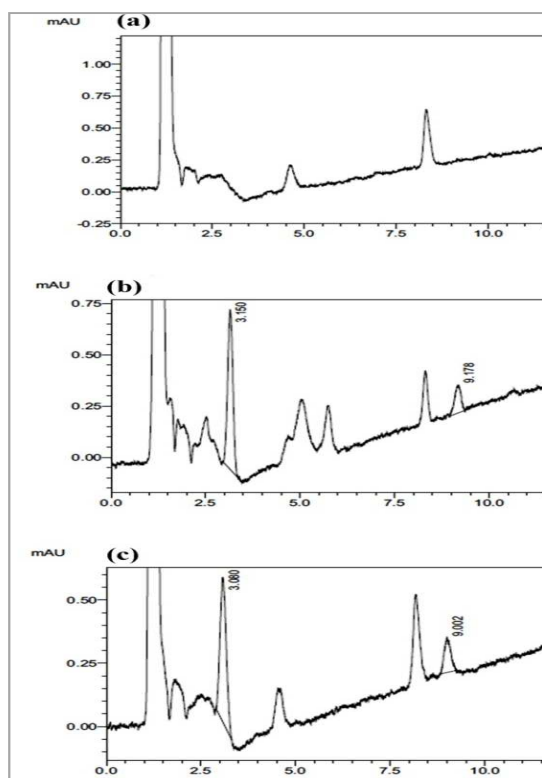
179 France), weighing approximately 20-22 g were housed in a controlled environment and were
180 fasted overnight for 12 hours, nevertheless they were allowed to have free access to water.
181 The animals were dosed via oral gavage with 5 mg/kg of fenofibrate. The compound was
182 orally administrated as suspensions of LIPANTHYL micronized.
183 Blood samples were collected at 0 h (pre-dose) 0.5, 1, 2, 3, 4, 6, 8, and 24 h post-dose from
184 the tail vein using a sparse sampling schedule consisting of two blood samples for each mouse
185 and 6 mice per time point. The samples were transferred to heparinized tube and centrifuged
186 at 10,000 rpm for 10min. The resulting plasma was separated and kept frozen at -80°C prior
187 analysis.

188 **3. RESULTS AND DISCUSSION**

189 *3.1 Method development*

190 The separation of fenofibric acid and carbamazepine (internal standard) from the
191 biological endogenous components in mouse plasma was found to be efficient and remarkably
192 within satisfactory range. Various combinations of mobile phase, isocratic and gradient
193 systems were investigated to achieve the optimum separation condition. We found that the
194 best wavelength for detection was 287nm.. The used mobile phase was Methanol and
195 ammonium acetate buffer (5 mM, (pH 3.3)). And optimal elution was attained by applying the
196 gradients steps (summarized in table 1) of solvent A (Methanol) and solvent B (ammonium
197 acetate buffer (5 mM, (pH 3.3))) at a flow rate of 1.5ml/min. The retention times of I.S and
198 fenofibric acid were 3 min and 9 min respectively.

199 Clear supernatants, which were immediately injected into HPLC, were obtained after
200 centrifugation of plasma samples that had been simply treated with Methanol and then cooled
201 to 0 °C by immersion in an ice bath for 30 minutes. The sample processing is consequently
202 convenient, inexpensive, and rapid. Furthermore, no evaporation step or a reconstitution phase
203 was needed. Otherwise during the method development, protein precipitation step was also
204 evaluated using diethyl ether but recovery, extraction rate and selectivity were found to be
205 unsatisfactory specially compared to the method using methanol which allowed a high
206 recovery and selectivity.



207
 208 **Fig. 1.** HPLC chromatogram of (a) blank mouse plasma. (b) plasma sample spiked with standards of
 209 carbamazepine (Rt-3 min) and fenofibric acid at a concentration of 0.64 μ g/ml (Rt-9 min). (c)
 210 plasma sample from a mouse, post dosing at 3h.

211 3.2. Selectivity study

212 Six different sources of blank plasma were screened in order to investigate any eventual
 213 interference from endogenous substances. Fig. 1 shows chromatograms obtained from the
 214 analysis of the extracted drug-free plasma, plasma sample spiked with fenofibric acid and the
 215 internal standard, and a plasma sample obtained from a mouse post dosing at 3h. The
 216 retention time for carbamazepine (internal standard) and fenofibric were, respectively, 3min
 217 and 9 min. With no endogenous compounds in the plasma eluted at the retention time of
 218 fenofibric acid and internal standard.

219 3.3. Linearity and lower limit of quantification

220 The peak areas ratio (Y) of fenofibric acid versus the internal standard (I.S) were proportional
 221 to the concentration of fenofibric acid (x) in plasma within the tested range (0.16 – 32 μ g/ml).
 222 Blank mouse plasma spiked with the corresponding standard solutions giving concentrations
 223 of 0.16, 0.32, 1.6, 3.2, 16 and 32 μ g/ml were analyzed. Excellent linear correlation was

224 obtained for all analytes over the entire studied concentration range. The standard equation
 225 was $y = 0,4316x - 0,0227$ and the correlation coefficient was $r^2=0.9999$. The analyte response at
 226 the lower limit of quantification (LLOQ) was found to be 5 times the response of the blank response
 227 (S/N ratio greater than 5) and was equal to $0.16\mu\text{g/ml}$ for fenofibric acid. The accuracy and
 228 precision at the LLOQ were acceptable, with 8.5% RE and 2.06% RSD (n=5).

229 3.4. Precision and accuracy

230 The intra- and inter- day precision and accuracy results are summarized in Table 2. The RSD
 231 and RE were less than 11% for the intra- and inter-run precision and accuracy assays. These
 232 described results inferred that the developed method was precise and accurate.

233 **Table 2.** Intra- and inter-day precision and accuracy for the determination of fenofibric acid in
 234 mouse plasma.

Spiked ($\mu\text{g/ml}$)	Intra-day precision and accuracy (n=5)			Inter-day precision and accuracy (n=15)		
	measured (mean \pm SD) $\mu\text{g/ml}$	RSD (%)	RE (%)	measured (mean \pm SD) $\mu\text{g/ml}$	RSD (%)	RE (%)
0.64	0.57 ± 0.03	4.82	10.00	0.58 ± 0.04	6.23	8.23
16	15.02 ± 1.02	6.82	5.70	16.13 ± 1.31	8.09	1.22
25.6	24.6 ± 2.69	10.92	3.51	25.75 ± 1.73	6.71	- 0.99

235 3.5. Recovery:

236 The extraction recovery was calculated for the three quality control concentration levels of
 237 fenofibric acid with three replicates for each level. The recovery of fenofibric acid at
 238 concentrations of 0.64, 16 and $25.6\mu\text{g/ml}$ were $93.39\% \pm 1.06$, $91.98\% \pm 6.83$, $92.49\% \pm 8.25$,
 239 respectively. The recovery of carbamazepine at the working concentration was $98,10 \pm 3,348\%$.

240 3.6 Stability:

241 The results of stability study, summarized in **Table 3**, showed that no significant degradation
 242 occurred to fenofibric acid during storage and extraction processes in mouse plasma samples.

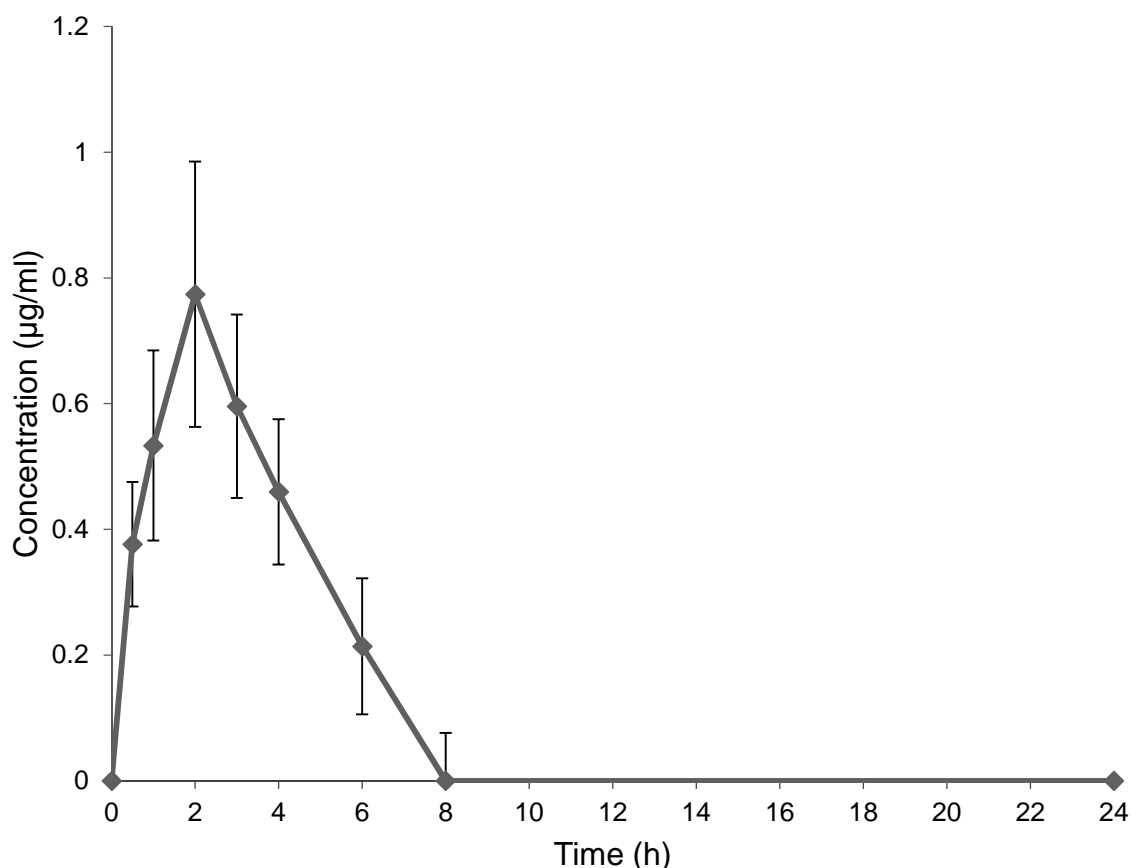
243 **Table 3.** Stability data of fenofibric acid in mouse plasma under different storage conditions
 244 (n=4)

storage conditions	Added concentration μg/ml	Found concentration μg/ml	Relative Error (%)	RSD (%)
Short-term stability (24 h, room temperature, n = 4)	0.64	0.710	-11.313	7.905
	16	15.645	1.833	1.464
	25.6	27.561	-8.084	6.027
Post-preparative stability (24 h, room temperature, n = 4)	0.64	0.652	-2.296	4.780
	16	16.177	-1.503	5.757
	25.6	26.223	-2.833	2.466
Long-term stability (30 d, -20 °C, n = 4)	0.64	0.689	-8.054	5.672
	16	15.608	2.070	1.782
	25.6	26.079	-2.271	2.313
Freeze and thaw stability (three cycles, -20 °C/room temperature, n = 4)	0.64	0.693	-8.769	6.803
	16	16.088	-0.942	7.392
	25.6	26.884	-5.426	4.421

245 **3.7. Application to pharmacokinetic study:**

246 After validation, the proposed method was applied for the determination of the fenofibric acid
 247 concentration in mouse plasma after oral administration of 5mg/kg fenofibrate to 24 male
 248 BALB/cJRj mice. Fig 2. Illustrates the mean plasma concentration/time curve of fenofibric
 249 acid. The plasma level of fenofibric acid reached a maximum concentration level (C_{max})
 250 equal to 0.831 ± 0.119 μg/ml, in (T_{max}) equal to 2h and the AUC_{0-24h} was 4.240 ±
 251 0.571 μg.h/ml. The obtained pharmacokinetic profiles were in close agreement with the PK
 252 profiles of fenofibric acid which were reported recently in literature [14-16].

253



254
255 **Fig 2.** Mean plasma concentration-time profiles of fenofibric acid in plasma following a
256 single oral dose of 5mg/kg fenofibrate to 24 male BALB/cJRj mice.

257 4. CONCLUSION

258 A HPLC method for the determination of fenofibric acid in mouse plasma has been developed
259 and completely validated for the first time. The preparation procedure of plasma samples was
260 suitable without any need to a reconstitution process or an extraction-evaporation phase. The
261 method was reproducible and the extraction recoveries were above 91.9% . Linear calibration
262 curve was obtained over the concentration range from 0,16µg/mL to 32µg/mL ($r^2=0,9999$) with
263 LLOQ of 0,16µg/mL The RSD in both intra-run and inter-run precision study was less than
264 11%. Besides, this study has shown that it was possible to overcome the problem of lower
265 volumes of plasma withdrawn by the developed sample preparation process and the drug
266 quantification method. Hence, the feasibility of mouse model to be used in pharmacokinetics
267 study of fenofibrate was confirmed as well as the extraction protocol with reference to the
268 results of analytical validation and successful application of the pharmacokinetic study. Finally,
269 this method may allow scientists to perform rapid and robust oral bioavailability studies of

270 fenofibrate in mice when it is used as drug model in some new drug delivery systems and
271 innovative formulations strategies.

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