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Citation for published version:
Tchoumtchoua, J, Halabalaki, M, Gikas, E, Tsarbopoulos, A, Fotaki, N, Liu, L, Nam, S, Jove, R & Skaltsounis, LA 2019, 'Preliminary pharmacokinetic study of the anticancer 6BIO in mice using an UHPLC-MS/MS approach', *Journal of Pharmaceutical and Biomedical Analysis*, vol. 164, pp. 317-325. https://doi.org/10.1016/j.jpba.2018.10.039

DOI:
10.1016/j.jpba.2018.10.039

Publication date:
2019

Document Version
Peer reviewed version

Link to publication

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Preliminary pharmacokinetic study of the anticancer 6BIO in mice using an UHPLC-MS/MS approach

Short title: Pharmacokinetic study of 6BIO in mice using UHPLC-MS/MS

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31 # Abstract

32 Indirubins represent a group of natural and synthetic products with bio-activities against numerous
33 human cancer cell lines acting by inhibiting protein kinases. The natural sources of indirubins are
34 plants of *Isatis sp.*, *Indigofera sp.*, and *Polygonum sp.*, recombinant bacteria, mammalian urine
35 and some marine mollusks. Specifically, the halogenated derivative 6-bromo indirubin-3′-oxime
36 (6BIO) possesses increased selectivity against GSK-3. However, to our knowledge, no analytical
37 method to determine 6BIO in biological fluids has been developed till now. Therefore, a rapid,
38 sensitive and high throughput UHPLC-MS/MS methods were developed and validated to evaluate
39 the concentrations of 6BIO in mice plasma. Plasma samples were pre-treated by protein
40 precipation using cold mixture of methanol: acetonitrile (9:1, v/v) and separations were carried out
41 on a Hypersil Gold C18 column (50 × 2.1 mm i.d.; 1.9 μm p.s.) using 0.1% acetic acid and
42 methanol as mobile phase at a flow rate of 500 mL/min in a gradient mode. For quantitation, a
43 hybrid LTQ-Orbitrap MS equipped with an electro-spray ionization source was used applying a
44 selected reaction monitoring (SRM) option. The monitored transitions were m/z 354.0 → 324.0
45 for 6BIO and 297.1 → 282.1 for afromorsin (used as the internal standard) in the negative mode.
46 Following the EMA, ICH and FDA guidelines for validation of analytical procedures, the assay
47 method was fully validated in terms of selectivity, linearity, recovery, matrix effect, accuracy,
48 precision, stability, and robustness. The validated methods were successfully applied to the
49 pharmacokinetic studies of 6BIO following an oral administration to mice at the dose of 50 mg/kg.
50 The results indicated that 6BIO possesses a T_{max} of 30 min, a half-life of 1 h, and low plasma
51 bioavailability.

52 Keywords: 6BIO, pharmacokinetics, UHPLC-Orbitrap MS, quantitation, plasma

53

54 # 1. Introduction

55 Indirubins are bis-indole alkaloid compounds occurring naturally in various indigo-containing
56 plants such as *Isatis spp* – Brassicaceae (1, 2), *Indigofera spp* – Leguminoseae (3), *Polygonum spp*
57 – Polygonaceae (4), *Strobilanthes spp* - (5). Besides their importance in the dye industry history,
58 these compounds display important biological activities with a particular focus on the inhibition
59 of protein kinases (6). A secondary natural source of indirubins are mollusk gastropods from
60 Muricidae family such as *Hexaplex trunculus* (7), *Plicopurpura spp* (8), and others (9). Plant
61 indirubin have been found as the active principle of the anti-leukemial Traditionnal Chinese
62 Medicine (TCM) *Danggui Longgui Wan* (10-11), with a particular action against Cyclin-
63 Dependent Kinases (12). On the other hand, the mollusk derived halogenated analogue, 6-bromo
64 indirubin (6BI) inhibits preferably the mamalian Glycogen Synthase Kinase-3 (GSK-3) (13, 14).
65 The anti-protein kinase activities of natural occurring indirubins have attracted the attention of the
66 scientific community this last decade, leading to a particular increase of interest in the study of the
67 biology and the chemistry of indirubins as reviewed in (15-17). Therefore, hundreds of indirubin
68 derivatives have been developed during the recent years, aiming towards the investigation and
69 amelioration of the activity, selectivity and drug-likeness of indirubins (18-22).

70 6-bromoindirubin-3′-oxime (6BIO) is a semi-synthetic analogue of 6BI that was developed as a
71 potent and selective GSK-3β inhibitor (23) and is currently considered as a prototype inhibitor of
72 that mammalian kinase. 6BIO has also been used to maintain pluripotency of mouse and human
73 embryonic stem cells through the reversible Wnt pathway activation, suggesting therefore its
74 applications in regenerative medicine (24). Besides GSK3β inhibition, 6BIO also inhibits
75 JAK/STAT3 signaling, induces apoptosis of melanoma cells *in vitro* and suppresses tumor growth

in vivo with low toxicity in a mouse xenograft model of melanoma (25). This anti-cancer like activity of 6BIO has been also investigated in the metastatic process of cancer cell lines *in vitro*: 6BIO inhibited adhesion, migration and invasion of a variety of metastatic cell types by inhibiting several kinase cascades such as PDK1, GSK3β and Jak/STAT3 (26). These results were confirmed by an *in vivo* experiment where 6BIO decrease significantly lung metastasis in the well-established 4T1 mouse model of aggressive breast cancer after a treatment at the dose of 1 mg/kg. Another reported mode of action of 6BIO is its potential to fight TRAIL (TNFα-related apoptosis-inducing factor) resistant cancer cells (27). Futhermore, 6BIO was recently found to be a full agonist of AhR, it participates, through AhR signaling, to the induction of Cytochrome P450-1A2 which is involved in one of the key metabolic functions of the liver (Briolotti et al., 2015). Moreover, the commercialization of 6BIO by Calbiochem (Merck-Millipore) and Santa Cruz Biotechnology under the name GSK-3 Inhibitor IX (CAS 667463-62-9) is a clear indication of its value as a tool for the exploration of physiological pathways. Despite the pleiotropic effects of indirubin derivatives and 6BIO in particular, scientific reports regarding the determination of 6BIO in biological fluids are very scarce.

To further explore the potential of 6BIO, a bioanalytical method was needed in view of pharmacokinetic studies with this compound. We therefore report here the development and validation of an UHPLC-ESI-HRMS/MS method for the determination of 6BIO in mice plasma. The use of UHPLC ensured the short analysis time (4.4 min), whereas the hybrid LTQ-Orbitrap Mass Spectrometer and the application of the SRM option offered high mass measurement accuracy and exclusion of any possible matrix interference and false positives from endogenous components having the same nominal mass. Overall, the developed methodology is suitable for high-throughput analysis requirements in the context of simple and efficient extraction with small

99 plasma volume, reduced analysis time and high sensitivity, all being necessary requirements for
100 accessing the pharmacokinetic profile of the drug. The developed methodology has been used to
101 access the pharmacokinetic profile of 6BIO after its administration in healthy mice.

102 2. Material and methods

103 2.1 Chemicals and reagents

104 6BIO (Figure 1) was synthesized as previously described (35). Afrormosin (I.S.) was isolated as
105 previously described (28). Glacial acetic acid and LC-MS grade water were purchased from Merk
106 (Damstart, Germany). LC-MS grade acetonitrile and methanol were purchased from Carlo Erba
107 (Val de Reuil, France).

108 2.2 Ultra-high performance liquid chromatography

109 The samples were analyzed on a UHPLC-LTQ-Orbitrap Discovery system (Thermo Scientific,
110 Bremen, Germany) consisted of an Accela UHPLC system coupled to a hybrid LTQ-Orbitrap
111 Discovery Mass spectrometer. An aliquot of 10 μL of sample was injected onto a C_{18} Hypersil
112 GOLD column (50 × 2.1 mm i.d., 1.9 μm particle size, Thermo Scientific) with a flow rate of 500
113 μL/min at room temperature. The LC linear gradient was increased from 10% B to 55% B in 2 min
114 (A, water with 0.1% acetic acid; B, acetonitrile), held at 55% B for 0.3 min, and then increased to
115 100% B in 1 min, restored to 10% in 0.1 min and held at 10% B for 1 min. The total run time was
116 4.4 min.

2.3 Mass spectrometry

Mass spectrometric detection was carried out on an LTQ-Orbitrap Discovery Mass Spectrometer (Thermo Finnigan) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z 354.0 → 324.0 and m/z 297.1 → 282.1 for 6BIO and Afrormosin respectively (Figure 2). The peak area ratio of 6BIO to the IS was used for the quantification of 6BIO in plasma samples. The optimized ionization parameters were as follows: ion spray voltage of 3.5 kV, temperature 300 ^{o}C, capillary voltage -50 V and tube lens -50 V. Two microscans were applied with a maximum injection time of 100 ms. Nitrogen was used as sheath gas (flow rate 50 arbitrary units) and auxiliary gas (flow rate of 10 arbitrary units). All samples were analysed in duplicate and data were acquired and processed using the Xcalibur 2.0 software.

2.4 Standard solutions, calibration standards and quality control samples

A stock solution of 6BIO was prepared by dissolving 5 mg of crystaline 6BIO in 5 ml of DMSO. The stock solution of the IS (Afrormosin) was prepared by dissolving 5 mg of the IS in 5 mL of methanol. Acetonitrile and water were used as solvent in the ratio of 1:1 (v/v) throughout the analysis. The stock solution of 6BIO was further diluted to give a series of sub-standard solutions with the concentrations of 50, 100, 250, 500, 1000, 1250, 1500 and 2000 ng/mL. The IS stock solution (1.0 mg/mL) was further diluted in methanol (HPLC grade) to yield a working standard solution of 500 ng/mL and subsequently stored at -20 ^{o}C pending analysis. The calibration standards of 6BIO were prepared by spiking 0.45 μL of 9 series of analyte-free mice plasma with 5 μL of the 9 series of appropriate 6BIO working standard solutions leading to plasma with a new

139 series of concentrations (5.0, 10, 25, 50, 100, 125, 150 and 200 ng/mL). LLOQ (5 ng/mL) and the
140 ULOQ (200 ng/mL) were prepared in pentaplicate. Similarly, the quality control (QC) samples
141 were prepared in pentaplicate using analyte-free mice plasma at concentrations of 7.5, 40 and 175
142 ng/mL.

143 2.5 Sample preparation

144 Protein precipitation was used for the treatment of plasma samples prior to the LC-MS analysis.
145 25 μL of the IS working solution was added to 225 μL of acetonitrile, vortexed for 30 s and
146 maintained at -20 ^{o}C. This new IS solution of 50 ng/mL (acetonitrile/methanol: 9/1, v/v) was added
147 to 50 μL of the plasma samples containing the analytes. The resulting suspension vortex-mixed
148 for 1 min, then centrifuged for 5 min (12000 rpm) and finally 10 μL of the supernatant were
149 injected into the UHPLC-LTQ-orbitrap system.

150 2.6 Method validation

151 The method was validated for selectivity, carry-over, linearity, accuracy, precision, dilution
152 integrity, robustness, matrix effect, extraction recovery and stability according to the International
153 Conference on Harmonization (ICH, 2005) Q2 (R1) procedures, the Bioanalytical Method
154 Validation Draft Guidance of the US Food and Drug Administration (FDA, 2013) and the
155 Guideline Bioanalytical method validation of the European Medicines Agency (EMA, 2011). The
156 peak area ratios of 6BIO to the I.S. of QC samples were interpolated from the calibration curve on
157 the same run to afford the concentration of 6BIO.

158 ### 2.6.1 Accuracy and precision

159 Precision and accuracy were evaluated by analyzing the samples at the three QC concentration
160 levels, along with the LLOQ and ULOQ at five replicates during three validation days. The
161 concentration of each sample was determined using the calibration curve prepared on the same
162 day. Precision was expressed using the coefficient of variation (CV, %) between the replicate
163 measurements. Accuracy was defined as the % relative error, .i.e. relative deviation in the
164 determined concentration of a standard from that of its nominal concentration expressed as a
165 percentage. The precision required was CV% within 15%, and accuracy were required not to
166 exceed ±15%.

167

168 ### 2.6.2 Specificity and selectivity

169 The interference of endogenous compounds in mice plasma was assessed as follows: Six blank
170 plasma from six different mice were processed without addition of 6BIO and I.S, and were
171 quantified using a valid calibration curve in order to test for possible interference at the retention
172 time of 6BIO and the IS. For all the rat plasma batches, the peak area measured in the blank plasma
173 sample had not to exceed 20% of the LLOQ peak area for 6BIO, and 5% for IS.

174 ### 2.6.3 Calibration model, LLOQ and ULOQ

175 The calibration model curves for 6BIO was assessed by analyzing nine calibration standard
176 samples in the range of 5.0 – 200 ng/mL in duplicate, employing the peak-area ratio of each analyte
177 to that of the IS *versus* its plasma concentration, A quadratic weighted ($1/x$) least squares regression
178 approximation has been selected as the most appropriate model.

The LLOQ was defined as the lowest concentration on the calibration curves of 6BIO measured with acceptable precision and accuracy (i.e. within ± 20% bias of the nominal value and with a %CV less than 20%) and a signal to noise ratio of at least 5. The ULOQ was defined as the highest concentration on the calibration curves of 6BIO.

2.6.4 Recovery (extraction efficiency)

Recovery of 6BIO after protein precipitation was estimated at the three QC concentration levels, as well as to the LLOQ and ULOQ by comparing the mean peak area ratios of the 6BIO to IS in pre- spiked extracted samples with post-spiked extracted blank samples, representing 100% recovery. In each case, five replicates were analyzed.

2.6.5 Matrix effect

The matrix effect was tested by comparing post-spiked blank samples to the corresponding standard saples at three QC concentration levels, as well as at the LLOQ and ULOQ. In each case, five replicates were analyzed. A value of 100% is indicative of no matrix effect, a value >100% suggests ionization enhancement and a value <100% suggests ionization suppression.

2.6.6 Carry over

For the evaluation of carryover effects, two plasma sample at the ULOQ level and two processed blank plasma were analyzed in consecutive LC-MS/MS runs. For acceptance, the peak areas of the blank sample should not exceed 20% of the peak areas obtained at the LLOQ, and 5% for the IS.

198 ### 2.6.7 Stability

199 In order to simulate the experimental conditions that an unknown sample may be exposed during
200 a routine analysis, free/thaw, autosampler, short term, and long term stabilities of samples were
201 carried out and always compared to that of a freshly prepared sets of calibrations and QC samples.
202 At each concentration level, the precision expressed as CV% had not to exceed 15%, and the
203 accuracy expressed as %Er had to be within ±15% of the nominal value.

204 #### 2.6.7.1 Three freeze - thaw cycles

205 Five replicates at the three QC concentration levels, LLOQ and ULOQ were subjected to three
206 freeze/thaw cycles (from freeze temperature of -20 ºC to room temperature) and were quantified
207 using the developed methodology.

208 #### 2.6.7.2 Short term stability

209 Five replicates at the three QC concentration levels, LLOQ and ULOQ were stored for 6 h at room
210 temperature before processing and analysis.

211 #### 2.6.7.3 Autosampler stability

212 Five replicates at the three QC concentration levels, LLOQ and ULOQ were processed and stored
213 at in the autosampler at 8 °C and protected from light, for 10 h and then analyzed.

214 #### 2.6.7.4 Long term stability

215 Long-term stability of samples was evaluated by analyzing QC, LLOQ and ULOQ samples that
216 were stored for 20 days at -80 °C.

217 ### *2.6.7.5 Robustness*

218 The robustness study was carried out to evaluate the influence of small deliberate variations of the
219 optimized chromatographic conditions. Three different parameters were altered, within a 5-10%
220 margin of the value used for the analysis, in order to evaluate the robustness of the proposed
221 methodology. These parameters were the flow rate, the column temperature and the percentage of
222 acetic acid in the aqueous phase. Thus, the values studied were 450, 500 and 550 µL/min for the
223 flow rate, 22, 24 and 26°C for the column temperature and 0.095, 0.1 and 0.105% for the
224 percentage of acetic acid in the aqueous phase. This study was performed at the LLOQ, ULOQ
225 and the QCs levels (n = 5). The evaluation of the results was accomplished by ANOVA.

226 ## 2.7 Pharmacokinetic study

227 The validated methodology was applied to determine the plasma concentration of 6BIO in mice
228 (n=3) up to 24h. 6BIO solutionin aqueous Solutol (30% w/v) was administered to mice at a single
229 dose of 50 mg/kg body weight (BW) by oral gavage. Blood samples were collected in
230 microcentrifuge tubes containing EDTA anticoagulants at intervals of 0, 5, 15, 30 min, and 1, 4,
231 6, 8, 12, and 24 h . Then, blood samples were centrifuged at 1500 rpm for 5 min at room
232 temperature. The obtained plasma samples were transferred into clean microcentrifuge tubes and
233 frozen at -80ºC.

234 Mean plasma concentrations of 6BIO after oral administration versus time curve were generated
235 in Microsoft Excel. The pharmacokinetic (PK) parameters were determined by non-compartmental
236 analysis of the individual plasma concentration profiles using the PKsolver (add-in program for
237 Microsoft Excel) The PK parameters determined were the maximum concentration (C_{max}), the time
238 to reach the maximum concentration (T_{max}), the terminal elimination half-life ($t_{1/2}$), the elimination

239 rate constant (*Ke*), the area under the curve from time zero to the last detectable sampling point
240 after administration ($AUC_{0\rightarrow last}$) and the area under the curve extrapolated to infinity (AUC_{0-}
241 $_{\infty}$),(calculated with the linear/log trapezoidal method), the mean residence time (MRT), the volume
242 of distribution at terminal phase (Vz), and the clearance (CL).

243 # 3 Results and Discussion

244 ## 3.1. Optimization of UHPLC-ESI MS/MS conditions

245 Despite the long history of indirubins in traditional, industrial and clinical practices, reported
246 methods for their quantitation in biofluids are very scarce. The first analysis of indirubins in rat
247 plasma was carried out using an HPLC-UV method (29). The limitations of this study were the
248 use of a low resolution and non-specific detection technique (UV), as well as the lengthy
249 chromatographic run times (env. 20 min). Hang et al. developed an HPLC-MS/MS method for the
250 quantitation of mesoindigo – an indirubin isomer – and its reductive metabolites in rat plasma (30),
251 with total analysis time reaching 30 min, which is a drawback in the analysis of a large number of
252 samples. Therefore, the main aim of this study was to develop an analytical methodology featuring
253 short analysis time for the quantitation of 6BIO in rat plasma. The UHPLC separation methodology
254 was employed due to its inherent capability of affording ultra-high resolution chromatographic
255 runs in a considerably short analysis time. The selected mobile phase (ACN and H_2O containing
256 0.1% FA) resulted in separation between 6BIO and the IS in 4.4-min run time (Figure 2). This
257 ultra-fast chromatographic separation of the analytes was combined with MS/MS spectral
258 detection, thus providing enhanced specificity and sensitivity. The MS/MS spectra from a Linear
259 Ion Trap based quantitative bioanalysis assay allows not only the separation and differentiation of

260 endogenous compounds in biological matrices from the drug compound of interest or its
261 metabolites, but also the elimination of any questions about false-positive data.

262 The first step of our method development was to acquire the high resolution Fourier transform full
263 scan and MS/MS spectra of 6BIO in both positive and negative mode. For this purpose, a 10 μg/mL
264 solution diluted in MeOH:H_2O (1:1, v/v) was directly infused into the MS system at a flow rate of
265 5 μL min^{-1}. The ESI interface parameters and the ESI probe position were also optimized for
266 obtaining the maximum abundance of precursor and product ions. In the full scan mode, 6BIO
267 exhibited two isotopic peaks: 353.9872 and 355.9852 (negative mode) vs 356.0027 and 357.0015
268 (positive mode). Both isotopes were taken into consideration to generated MS/MS spectra as
269 summarized in table 1.

270 Linear Ion Trap full scan and MS/MS spectra of 6BIO were further acquired using the same
271 conditions utilized for Orbitrap acquisition. For MS/MS experiments, the collision energy voltage
272 for the effective fragmentation of the selected precursor ions of 6BIO was optimized over the range
273 of 5-35% with a 2% step, and it was shown that 17% provided optimum fragmentation of 6BIO in
274 negative mode and 19% in positive mode (Figure 2).

275 In both Orbitrap and Linear Ion Trap acquisitions, negative mode provided greater S/N ratio as
276 compared to positive mode. However, the Linear Ion Trap rather than the Orbitrap provided more
277 stable measurement since CV % values obtained employing Orbitrap analysis were in general >
278 35% and usually 10-fold higher than those in the case of Linear Ion Trap. Furthermore, the CV %
279 and RE % remained the same whether selecting either isotope ion or both. Considering all the
280 above, the Ion Trap operation at negative mode was selected for the analyses with a CID of 17%
281 for the selected 354 ion of 6BIO. The IS MS/MS spectrum was acquired using a 35% CID as

282 previously reported (40). Product ions of 6BIO and IS after fragmentation with the Linear Ion trap
283 are shown in figure 3.

284 The chromatographic conditions were optimized for the rapid and efficient separation of 6BIO and
285 IS from plasma components. For this reason, a 5 cm length C18 silica column was chosen with
286 acetonitrile as the organic mobile phase over methanol as this combination gave the best results in
287 terms of peak shape (width, symmetry, sharpness) and accelerate the analysis time. Further
288 optimization was achieved by adding 0.1% acetic acid in the aqueous mobile phase enhancing
289 therefore the ionization efficiency in comparison to various percentages of formic acid. Under the
290 optimized chromatographic conditions 6BIO was eluted at 3.08 min. and afromorsin (IS) at 2.52
291 min (Figure 4).

292 3.2. Sample preparation

293 Numerous methods were evaluated for the sample preparation procedure. Specifically, (i) protein
294 precipitation with cold mixture of acetonitrile/methanol at various ratios (1/9, v/v), (3/7, v/v), (1/1,
295 v/v), (7/3, v/v), and (9/1, v/v); (ii) liquid-liquid extraction, and (iii) SPE extraction. Amongst all,
296 protein precipitation with cold acetonitrile/methanol (9:1, v/v) was chosen as it presented the
297 highest recovery factor, the least matrix effect and was the less time consuming presenting also
298 adequate sensitivity for the PK study of 6-BIO concentration after its administration to mice.

299 3.3. Choice of the IS

300 Afromorsin was selected as the IS mainly due to the fact that it is a readily available, inexpensive,
301 not harmful and stable substance that could be ionized in the negative ionization mode. Moreover,
302 afromorsin was eluted before 6BIO and was well separated from 6BIO without interference from

303 endogenous compounds in rat plasma, whereas its chromatographic characteristics were
304 appropriate.

305 3.4. Method validation

306 *3.4.1. Selectivity*
307

308 The selectivity of our method refers to its ability to select and determine particular 6BIO and IS in
309 plasma without interfering with the other metabolites. Therefore five plasma sample at the LLOQ
310 were analyzed. The selectivity precision (CV %) was 9.62%, and the accuracy (RE %) was 1.14%
311 (Table 2). Since the precision was below 20%, and the accuracy was within ±20% at the LLOQ,
312 this analytical method for the quantification of 6BIO in rat plasma was shown to be selective.

313 *3.4.2. Specificity*
314

315 The specificity of the proposed analytical methodology was assessed by the analysis of six blank
316 drug-free plasma samples. Analysis of these blank samples under the same conditions revealed no
317 interference peaks from endogenous plasma components at the retention times of 6BIO and IS; the
318 peak areas measured in the blank plasma sample were equal to 0.00% and, hence, less than 20.0%
319 of the LLOQ average peak area, indicating that this analytical method is specific for 6BIO and
320 suitable for bioanalysis. The representative chromatograms of blank rat plasma and plasma spiked
321 with 6BIO at LLOQ and ULOQ and IS are shown in Figure 4.

322 *3.4.3. Calibration range, calibration model, and LLOQ*
323

324 Having no idea of the concentration of samples to be analyzed, and in order to avoid working with
325 either a very large or a very narrow calibration curve, we firstly performed a preliminary
326 calibration curve of 6BIO alone over the range 1 – 5000 ng/mL along with representative plasma

327 samples of the PK study. After analysis of the data, our concentration range was narrowed to 5 –
328 200 ng/mL; final calibration curves were constructed by plotting the peak area ratios of 6BIO to
329 IS of plasma and calibration standards versus nominal concentrations of 6BIO. The calibration
330 model was selected based on the analysis of the data by linear and non-linear regression as well as
331 with and without weighting. The curve fitting was based on the simplest model that exhibited the
332 highest correlation coefficient along with the lowest RE% on the back-calculated values.
333 Calibration curves of five different lots of plasma calibration standards were constructed (Table
334 3).

335 The best fit and least square residuals for the calibration curves were achieved employing a
336 quadratic model with 1/x weighting factor. The correlation coefficient (r^2) of the calibration curve
337 was > 0.998, indicating good correlation. The back calculated values obtained are within the
338 proposed 15% margin of the nominal concentrations indicating the adequacy of the proposed
339 model. The LLOQ of 6BIO with the proposed method was determined to be 5 ng/mL fulfilling the
340 specification that RE % and CV % should fall into the 20% margin and the S/N the analyte should
341 be at least 5 (at least 5 times the signal of a blank sample).

342 In order to clarify if the calibration curves were the same an extra sum of squares F-test has been
343 carried out. The results show that the calibration curves obtained do not differ statistically and
344 therefore each one could be substituted either by one other in the set or could be described by one
345 common curve. The consensus curve can be expressed by the following equation:

346 $$Y=0.00015(\pm 0.00014)*X^2+0.97(\pm 0.02)*X+0.20(\pm 0.39)$$

347 The numbers in parentheses denote the standard error. The back calculated values calculated by
348 the equation exhibit RE % values less than 15% for all points whereas the coefficient of the
349 determination is better than 0.998.

350 *3.4.4. Accuracy and precision*
351
352 The intra- and inter-day precision and accuracy were evaluated at the three QC levels as well as at
353 the LLOQ and ULOQ levels (Table 4). As shown in Table 4 the intra-day precision does not exceed
354 10.11 % whereas the inter-day was better than 9.62 % in every case. The RE %, indicating the
355 accuracy of the method does not exceed 13.29%. Therefore, this bio-analytical method proved to
356 be precise and accurate.

357 *3.4.5. Recovery and matrix effect*
358
359 In order to evaluate the recovery of 6BIO, twenty-five blank mouse plasma samples from five
360 different animals were processed without addition of 6BIO and concomitantly they were spiked
361 with 6BIO at the LLOQ, ULOQ, and QCs concentration levels (post spiked samples). The
362 responses obtained represented the 100% recovery reference. The extraction recovery was
363 calculated by dividing the peak area ratio of the post-spiked samples by the corresponding peak
364 area ratio of the pre-spiked samples (spiked before protein precipitation). Similarly, the potential
365 ion suppression or enhancement due to mouse plasma components (matrix effect) was evaluated
366 by dividing the peak area ratio of the post-spiked plasma samples by the corresponding peak area
367 ratio of working standard solutions diluted in MeOH:H2O (1/1, v/v). The extraction recovery of
368 6BIO was between 97.1 and 124.4 % across the entire range (5 - 200 ng/mL), while the matrix
369 effect was between 73.8 and 85 % for 6BIO (Table 5).

370 *3.4.6. Carry-over*
371
372 The impact of the carry-over on blank mouse samples following the highest calibration sample
373 was assessed for both 6BIO and IS. The average carry-over was 1 % (below 20%) for 6BIO and
374 0.04 % (below 5%) for the IS, indicating that the carry-over has no impact on the results.

375 *3.4.7. Stability*
376
377 No significant degradation have been observed under the stress condition imposed as described
378 during the stability study (Table 6). Therefore, no special precautions are advised besides the ones
379 described.
380
381 *3.4.8. Robustness*
382
383 Upon variation of the flow rate, the column temperature, and the percentage of acetic acid in
384 aqueous phase, the retention time shift of 6BIO and IS remained statistically not significant at the
385 95% level using ANOVA.

386 ## 3.5 Pharmacokinetic Study
387
388 This validated method was successively applied to a pharmacokinetic study of 6BIO in mice. The
389 mean plasma concentration – time curve after a single oral administration of 6BIO at 50 mg/kg
390 BW is shown in Figure 5.

391 The main pharmacokinetic parameters of 6BIO calculated using PKsolver software are
392 summarized in Table 7. After administration of 6BIO, the mean maximum concentration (C_{max})
393 was 118.2 ± 58.7 ng/mL, and the area under the concentration-time curve (AUC_{0-Inf}) was 155.32
394 ± 34.99 ng*h/mL. The plasma concentration of 6BIO decreased rapidly and was eliminated from
395 the plasma with a half-life time ($t_{1/2}$) of 0.72 ± 0.09 hours.

396 This study with an oral administration of 50 mg/kg BW validated the bioanalytical method
397 developed and could be suitable to detect 6BIO levels in mouse plasma. Overall, low
398 bioavailability and rapid decrease of 6BIO concentrations in plasma is observed. The low
399 bioavailability could be attributed to the low solubility of 6BIO (sparingly soluble in aqueous

400 buffres, 0.3 mg/ml in a 1:2 solution of DMSO:PBS (pH 7.2)). The rapid decline of plasma
401 concentrations might result from rapid distributions to other tissues. In future PK studies, we will
402 investigate the distribution of 6BIO to other tissues after oral administration and the PK after
403 intravenous injection can improve the plasma bioavailability of 6BIO.

404 4. Conclusion

405 The described UHPLC-ESI-MS/MS analytical methodology enables the rapid and selective
406 determination of 6BIO in mouse plasma. The developed method is robust and presents high
407 sensitivity, accuracy, precision, recovery, and stability. A small amount of plasma is required using
408 this method (45 μL), making it applicable for many bio-analytical studies which generally present
409 limited amount of sample. A time-saving one-step protein precipitation for sample pretreatment,
410 combined to the rapid analysis time (4.4 min) render this method readily applicable in a further
411 clinical study, comprising a large amount of samples and need for high-throughput method. The
412 method was therefore applied to a preliminary pharmacokinetic study of 6BIO after oral
413 administration of 50 mg/kg in mice. The pharmacokinetic profile of 6BIO was characterized for
414 the first time and this showed a low bio-availability and a rapid elimination of 6BIO from the
415 systemic circulation in mice.

416 Conflict of interest

417 Authors declared no conflict of interest.

418 # Acknowledgements

419 ……………………………………………

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517 successively.

518 Figure 4: Representative segmented MRM chromatograms of: (A) blank mouse plasma sample,
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520 plasma spiked with 6BIO (200 ng/mL) and IS (50 ng/mL), and (D) plasma sample collected 5 min
521 after oral administration of 6BIO (50 mg/kg) to mice.

522 Figure 5: Mean plasma concentration time profile of 6BIO in mice (n = 3) following oral
523 administration (50 mg/kg BW). For representative reason, time points have been constrained up to
524 6h.

525

526

527 Table 1: High resolution precursor and product ions of 6BIO in positive and negative ESI-Orbitrap

Precursor ions		CID	Product ions
Negative	353.9872	17	323.9893
	355.9852	17	325.9872
Positive	356.0027	20	326.0045
	357.0015	20	328.0035

528

529

530 Table 2: Selectivity test at the LLOQ for 6BIO (n = 5)

Run	Replicate	Nominal level (ng/mL)	Calculated value (ng/mL)	Mean	SD	RE %	CV %
1	1	5	4.893	5.1	0.49	1.14	9.62
	2	5	4.491				
2	1	5	4.961				
	2	5	4.556				
3	1	5	5.882				
	2	5	5.873				
4	1	5	4.794				
	2	5	4.780				
5	1	5	5.210				
	2	5	5.130				

SD: standard deviation

RE %: deviation of mean from the nominal value

CV %: coefficient of variation

531

532

533 Table 3: Calibrators and calibration curve parameters

Run	Nominal values (ng/mL)									r^2
	5	**10**	**25**	**50**	**75**	**100**	**125**	**150**	**200**	
1	4.9	9.2	24.4	51.0	71.7	105.3	122.9	151.2	207.7	0.998
	4.5	9.8	24.4	52.2	70.6	104.2	124.0	146.0	212.6	
2	4.6	9.4	22.6	49.7	78.5	101.6	125.0	150.2	220.7	0.999
	4.9	10.6	23.0	48.5	77.2	98.0	129.1	147.8	202.7	
3	4.3	12.1	25.2	49.9	84.5	99.6	116.9	137.1	206.6	0.992
	4.3	10.0	24.1	53.6	84.4	97.7	115.0	135.6	219.4	
4	4.7	9.9	26.3	52.1	80.6	99.1	118.7	129.9	204.8	0.998
	6.1	10.5	25.0	48.8	76.0	95.3	123.3	123.1	200.3	
5	5.4	10.4	23.7	52.3	84.7	92.3	120.4	146.5	201.8	0.997
	5.1	9.7	25.4	51.9	80.7	93.8	119.1	148.2	209.0	
Mean	**4.9**	**10.1**	**24.4**	**51.0**	**78.9**	**98.7**	**121.4**	**141.6**	**208.6**	
SD	0.56	0.83	1.13	1.68	5.09	4.25	4.21	9.60	7.07	
RE %	-2.32	1.44	-2.39	1.98	5.19	-1.32	-2.85	-5.62	4.28	
CV %	11.47	8.14	4.61	3.29	6.45	4.31	3.46	6.78	3.39	

534

535 *Run 1*: Y1 = 0.0025 + 0.011*X - 1.33e-005*X^2 $r^2 = 0.998$

536 *Run 2*: Y2 = -0.0052 + 0.013*X - 8.22e-006*X^2 $r^2 = 0.999$

537 *Run 3*: Y3 = 0.0002 + 0.008*X - 2.04e-006*X^2 $r^2 = 0.992$

538 *Run 4*: Y4 = 0.0123 + 0.007*X - 1.00e-006*X^2 $r^2 = 0.998$

539 *Run 5*: Y5 = 0.0002 + 0.007*X + 2.68e-007*X^2 $r^2 = 0.997$

540

541 Table 4: Within- and between-series precision (CV %), and accuracy (expressed as RE %) of

542 LLOQ, ULOQ and QC samples

Level	Found	Day1	Day 2	Day 3			Overall
		n =5	n = 5	Run 1 (n = 5)	Run 2 (n = 5)	Run 3 (n = 5)	n = 25
LLOQ (5 ng/mL)	Mean	**5.1**	**5.1**	**5.0**	**4.9**	**5.0**	**5.1**
	SD	0.49	0.40	0.35	0.14	0.21	0.06
	RE %	1.14	2.51	0.77	2.00	1.00	1.39
	CV %	9.62	7.79	7.02	2.89	4.29	1.14
LQC (7.5 ng/mL)	Mean	**7.6**	**7.8**	**7.4**	**6.5**	**8.4**	**7.3**
	SD	0.59	0.29	0.74	0.47	0.85	0.57
	RE %	1.83	4.10	1.93	13.29	12.06	2.33
	CV %	7.70	3.69	10.11	7.24	10.08	7.83
MQC (40 ng/mL)	Mean	**43.1**	**39.1**	**44.2**	**44.5**	**42.6**	**42.7**
	SD	1.89	1.37	1.44	1.50	2.42	2.16
	RE %	7.83	2.23	10.47	11.31	6.46	6.75
	CV %	4.39	3.51	3.27	3.37	5.69	5.05
HQC (175 ng/mL)	Mean	**185.3**	**175.1**	**185.2**	**186.5**	**181.8**	**183.0**
	SD	5.77	8.67	12.94	12.74	5.32	5.32
	RE %	5.86	0.05	5.85	6.54	3.86	4.59
	CV %	3.12	4.95	6.99	6.83	2.92	2.90
ULOQ (200 ng/mL)	Mean	**200.7**	**199.8**	**194.9**	**206.9**	**195.0**	**200.3**
	SD	15.44	9.53	0.74	7.85	2.83	0.64
	RE %	0.35	0.09	2.6	3.43	2.50	0.13
	CV %	7.69	4.77	0.38	3.79	1.45	0.32

543

544

545 Table 5: Recovery of 6BIO in mouse plasma and matrix effect (n = 5)

Level	ng/mL	Matrix effect (%)	SD	RE %	CV %	Recovery (%)	SD	RE %	CV %
LLOQ	**5**	**73.8**	9.36	26.25	12.69	**98.5**	3.42	1.54	3.48
LQC	**7.5**	**83.6**	13.28	16.45	15.89	**109.0**	12.66	9.02	11.6
MQC	**40**	**85.0**	6.53	15.00	7.68	**97.1**	4.80	2.86	4.94
HQC	**175**	**82.8**	3.57	17.25	4.31	**94.6**	7.65	5.41	8.08
ULOQ	**200**	**79.5**	2.57	20.50	3.23	**124.4**	2.83	24.36	2.28

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547

548 Table 6: Stability results of 6BIO in mice plasma under various conditions (n = 5)

Level	Spiked Conc. (ng/mL)	Freeze-thaw (-20 ºC to 25 ºC)			Short-term (6h, 25 ºC)			Autosampler (10h, 8 ºC)			Long term (15 days, -80 ºC)		
		Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)	Mean conc. Found (ng/mL)	RE (%)	RSD (%)
LLOQ	5	5.1	10.00	9.80	4.95	5.00	6.06	5.12	12.00	5.86	5.03	3.00	9.94
LQC	7.5	7.7	2.67	9.09	6.9	8.00	10.14	7.40	1.33	13.51	7.55	0.67	10.60
MQC	40	42.5	6.25	5.53	44.0	10.0	8.07	43.5	8.75	10.46	40.50	1.25	14.57
HQC	175	180.5	3.14	5.82	173.5	0.86	3.07	173.5	0.86	4.32	170.5	2.57	5.87
ULOQ	200	201.1	0.55	3.73	205.5	2.75	3.16	199.5	0.25	3.76	195.0	2.50	3.85

551 Table 7: Non-compartmental pharmacokinetic parameters of 6BIO in plasma after a single oral
552 dose of 50 mg/kg BW to mice (n = 3).

Parameter	**Unit**	**Mean values**	**SD**
T_{max}	h	0.50	0.0
C_{max}	ng/mL	118.2	58.7
Elimination rate constant (Ke)	1/h	0.97	0.12
Terminal elimination half-life (t1/2)	h	0.72	0.09
AUC 0-t	ng/mL*h	155.3	35.0
AUC 0-inf	ng/mL*h	157.6	36.2
MRT 0-inf	h	0.98	0.16
Vz/F	(mg/kg)/(ng/mL)	0.35	0.13
Cl/F	(mg/kg)/(ng/mL)/h	0.33	0.09

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