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1 **Pharmacokinetic Report**

2

3 **Pharmacokinetics, metabolism and excretion of celecoxib, a selective cyclooxygenase-2**

4 **inhibitor, in horses**

5

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22

23 **Abstract**

24 Celecoxib, a nonsteroidal anti-inflammatory drug is frequently used to treat arthritis in  
25 humans with minimal gastrointestinal side effect compared to traditional NSAIDs. The  
26 primary aim of this study is to determine the pharmacokinetic profile of celecoxib – a  
27 selective cyclooxygenase-2 (COX-2) inhibitor in horses. Six horses were administered a  
28 single oral dose of celecoxib at 2 mg/kg (body weight). After oral dosing, the drug reached a  
29 maximum concentration (mean  $\pm$  SD) in blood of  $1088 \pm 324$  ng/mL in 4.58 h. The  
30 elimination half-life was  $13.60 \pm 3.18$  h and the area under the curve was  $24142 \pm 1096$   
31 ng.mL/h. The metabolism of celecoxib in horses was via a single oxidative pathway in which  
32 the methyl group of celecoxib is oxidised to a hydroxymethyl metabolite and is further  
33 oxidised to form a carboxylic acid metabolite. Celecoxib is eliminated mainly through faeces  
34 as unchanged drug and as metabolites in urine. Therefore, instructions for a detection time  
35 following therapeutic dosing of celecoxib can be set by the racing practitioner and  
36 veterinarians to control illegal use in horse racing based on the results of this study.

37

38

39 *Keywords:* Celecoxib; Elimination; Horses; Metabolism; Pharmacokinetics.

## 40 **Introduction**

41 Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]  
42 benzenesulfonamide is the first COX-2 inhibitor to be developed as an analgesic with less  
43 toxicity on the gastrointestinal tract (Noble et al., 2000). Despite celecoxib has been clinically  
44 proven to be effective as a therapy for many species of animals (Zhang et al., 2000; Paulson et  
45 al., 2001; Störmer et al., 2003; Ma et al., 2015) there is limited data reported in horses  
46 although being banned as doping agent by horse racing authorities (The International  
47 Federation for Equestrian Sports, n.d.). A GC/MS method using TMS derivatization and an  
48 LC/MS study have been reported to characterize the metabolites of celecoxib in horse urine  
49 (Dirikolu et al., 2001; De Kock et al., 2005). However, the urine samples were collected for a  
50 short duration and a clear determination of detection time could not be established in their  
51 reports. Further, no detailed study to show data for the drug pharmacokinetics and detection  
52 time in horses is available. As such, this study was designated to characterise the plasma  
53 pharmacokinetics, metabolism pathways and elimination route of celecoxib given orally to  
54 horses, which assess the clinical and animal welfare implications and derive regulatory advice  
55 for horse racing industry.

56

## 57 **Materials and methods**

### 58 *Animals*

59 Six adult horses (males, male castrates and females) aged 12 to 18 years and weighing  
60 approximately 480 kg were used in the study. All animals were tagged, dewormed and housed  
61 in air-conditioned stable barns. The horses were given regular walking exercise for 30  
62 minutes twice a day. Regular physical examination was performed on each horse by the local  
63 hospital veterinarian working adjacent to this facility. The animals were fed twice a day with  
64 hay, alfalfa and grains. They had free access to water. None of the horses had any disease

65 history in their stable records and they were not treated with NSAIDs or other anti-  
66 inflammatory drugs for at least 30 days prior to this administration study. The animals were  
67 fasted overnight prior to treatment and food was supplied after 2 hours of the first blood  
68 sample collected after drug administration. The study was approved by the Animal Ethics  
69 Committee of the Central Veterinary Research Laboratory in Dubai, U.A.E.

70

#### 71 *Experimental chemicals*

72 Celecoxib and zaleplon were kind donations by Ipca Laboratories Limited, Indore,  
73 India, both having an assay purity of  $\approx 99.8\%$ .  $\beta$ -glucuronidase from *Helix pomatia* (type  
74 HP2) and NADPH were purchased from Sigma Chemical Co. Ltd.. Hydroxymethyl  
75 celecoxib and celecoxib carboxylic acid were obtained from Alsachim (France). All organic  
76 solvents were either HPLC grade or LC/MS grade and purchased from Fisher Scientific.  
77 Horse liver microsomes were bought from Xeno Tech U.S.A for in vitro studies.

78

#### 79 *Treatment and sample collection*

80 The study involved administration of a single oral solution dose of celecoxib at 2  
81 mg/kg body weight (bwt) to six horses using a naso-gastric tube. The administration was done  
82 in the presence of a clinical veterinary doctor. The drug was mixed with 250 mL of drinking  
83 water and poured into the tube using a funnel. The funnel was rinsed with another 100 mL of  
84 drinking water to ensure complete delivery of the drug into the horse stomach. Prior to drug  
85 administration an 18-gauge catheter was placed either into the right or left jugular vein for the  
86 collection of blood samples. Blood samples were collected into heparinised tubes at 0, 5, 10,  
87 15, 30, 45 minutes and at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours post  
88 medication. The samples were immediately centrifuged, plasma separated and kept frozen at -  
89 20°C until use for drug analysis. A custom made diaper was tied to the animal for the

90 collection of urine and faeces. Urine and faeces samples were collected whenever the animal  
91 disposed for seven days after administration and were pooled into morning and evening urine  
92 and faeces composites and stored at - 20°C until used for drug analysis.

93

#### 94 *Plasma sample analysis*

95 Plasma samples were analysed by use of liquid chromatography- mass spectrometry  
96 (LC/MS). A previously validated solid phase extraction (SPE) method was carried out using  
97 automated extraction modules (RapidTrace, Biotage) and Waters SepPak C18 cartridge was  
98 used for the determination of celecoxib. Briefly, the C18 cartridge was sequentially  
99 conditioned with 3 mL of methanol and 0.1 M KH<sub>2</sub>PO<sub>4</sub> (pH 6.0). A volume of 1 mL plasma  
100 was allowed to pass through the column. Then the column was rinsed sequentially with 1 mL  
101 0.1 M KH<sub>2</sub>PO<sub>4</sub> : methanol (90:10) and 1 mL of 1.0 M acetic acid. Following drying for 2  
102 minutes, the drug and its metabolites were eluted with 4 mL dichloromethane. The organic  
103 solvent was dried under a gentle flow of nitrogen on a TurboVap (Biotage) at 40 °C. The  
104 residue was reconstituted in 50 µL of mobile phase. In addition to administration samples,  
105 quality control samples with the drug spiked at different concentrations (1, 10, 100, 500 and  
106 1000 ng/mL) were also assayed with each set.

107 A stock standard was prepared by dissolving 1 mg of celecoxib in 100 mL of acetonitrile. The  
108 stock standard was further diluted by serial dilution to prepare standard solutions of 1, 100  
109 and 500 ng/mL. Similarly 1 mg of zaleplon was dissolved in 100 mL of methanol. This was  
110 further diluted to 1 µg/mL and used as internal standard. Calibration standards ranged from 1  
111 to 1000 ng/mL were prepared by adding appropriate volumes of the standard drug solutions to  
112 drug free plasma. Zaleplon was used as the internal standard. A volume of 200 µL (1 µg/mL)  
113 of the internal standard was added to the calibration and test sample and extracted as  
114 mentioned above.

115 The LC/MS system consisted of a Surveyor LC pump and auto sampler (Thermo Finnigan)  
116 connected to a TSQ Access (Thermo Finnigan) mass spectrometer. Chromatographic  
117 separation was performed using a Thermo Hypersil C18 column (100 x 2.1 mm ID., 5 µm  
118 particle size) using a mobile phase consisting of acetonitrile and 1% formic acid in water, run  
119 in a gradient mode. The mass spectrometer was operated in the negative ion mode performing  
120 product ion scans (m/z 380 → 316), (m/z 396 → 302) and (m/z 410 → 366) for celecoxib,  
121 hydroxymethyl celecoxib and celecoxib carboxylic acid, respectively. Zaleplon was scanned  
122 in the positive ion mode for the transition (m/z 306 → 264).

123 The present study was able to examine the pharmacokinetics and characterize the metabolic  
124 disposition of celecoxib after a single oral dose to horses, applying the proposed highly  
125 selective and sensitive LC/MS with a limit of quantitation (LOQ) of 1 ng/mL and a limit of  
126 detection (LOD) of 500 pg/mL. No interfering peaks for celecoxib were observed in the  
127 chromatograms of blank plasma from the horses used in the study. The intra-day and inter-day  
128 assay precision and accuracy for low, medium and high (10, 100, 200 ng/mL) concentration  
129 of celecoxib in horse plasma showed a coefficient of variation percentage (CV%) ranging  
130 from 5.6 to 9.8 and 4.9 to 8.0, respectively.

131

132

### 133 *Pharmacokinetic analysis*

134 Pharmacokinetic parameters were determined for each animal individually utilizing  
135 application of the trapezoidal rule for measurements of plasma drug concentration versus time  
136 curve to achieve the non-compartmental methodology (Gibaldi & Perrier, 1982; Martinez,  
137 1998; Gabrielsson & Weiner, 2012) using a computerized pharmacokinetic and drug  
138 disposition program (Kinetica Version 5.1 SP1, Thermo). For each parameter, the mean and  
139 standard deviation (SD) were obtained from the calculation results of six animals ( $n = 6$ ).

140 In order to estimate the celecoxib dose for horses from an efficacious dose in human, the  
141 following equation was applied (Toutain & Bousquet-Mélou, 2004):

$$142 \quad Dose_{horse} = \frac{Dose_{human} \times CL_{horse}}{CL_{human}} \quad (1)$$

143 In addition, determinations of an effective plasma concentration (EPC), irrelevant plasma  
144 concentration (IPC), safety factor (SF) and detection time (DT) were based on the following  
145 equations (Toutain & Lassourd, 2002):

$$146 \quad EPC = \frac{\text{standard dose (per dosing interval)}}{\text{plasma clearance (per dosing interval)}} \quad (2)$$

$$147 \quad IPC = \frac{EPC}{SF} \quad (3)$$

$$148 \quad IPC = C_{max} \times e^{(-\beta \times DT)} \quad (4)$$

149

#### 150 *Determination of metabolites*

151 Urine samples were pooled as '0' hour (control), 1-12, 12-24 hours and so on up to  
152 192 hours after administration. An aliquot of 10 mL of each urine sample was adjusted to pH  
153 5.2 and approximately 10,000 Fishman units of  $\beta$ -glucuronidase was added and incubated at  
154 37 °C overnight. Another aliquot of 10 mL urine was processed simultaneously without  
155 enzyme hydrolysis. Similarly an amount of 10 grams of faeces was weighed and mixed  
156 thoroughly with 10 mL of distilled water. The slurry is then centrifuged at 4000 *g* for 15  
157 minutes. A volume of 7 mL of this clear liquid was adjusted to pH 5.2 and enzyme  
158 hydrolysed overnight at 37 °C.

159 The enzyme hydrolysed urine and faeces samples were adjusted to pH 6.0 and again  
160 centrifuged for 15 minutes. To 5 mL of each sample 200  $\mu$ L (1 $\mu$ g/mL) of internal standard  
161 was added and subjected to solid phase extraction as described above. The final eluent was  
162 dried and reconstituted with 50  $\mu$ L of the mobile phase for LC/MS analysis.

163



164 *In vitro* metabolism of celecoxib

165 To characterize the *in vitro* metabolic pathway, celecoxib was incubated with horse  
166 liver microsomes. In this study, all the incubations were performed in duplicates in a shaking  
167 water bath at 37°C. The experimental protocol is as follows: To 5 µL of celecoxib (1mM) is  
168 added 432 µL of 0.1M sodium phosphate buffer (pH 7.4) and 13 µL of the protein (0.5  
169 mg/mL), premixed and allowed to stand at room temperature for 3 minutes. The incubation  
170 was commenced by the addition of 10 mM NADPH (50 µL) and allowed to react for 30  
171 minutes with constant gentle shaking. After incubation the reaction was quenched by addition  
172 of 100 µL of ice cold methanol. The resulting mixture was then extracted with  
173 dichloromethane, centrifuged and the organic phase was dried and reconstituted with 50 µL of  
174 the mobile phase for LC/MS analysis.

175

176 **Results**

177 The pharmacokinetic parameters of celecoxib after an oral dose of 2 mg/kg bwt are  
178 shown in Table 1. The results show that after oral administration of celecoxib the time to  
179 reach peak plasma concentration ( $t_{max}$ ) was  $4.58 \pm 1.62$  hours and the rate of elimination was  
180 calculated to be  $0.05 \pm 0.01 \text{ hr}^{-1}$ , while the terminal half-life ( $t_{1/2}$ ) was estimated to be  $13.60$   
181  $\pm 3.18$  hours. The mean  $\pm$  standard deviation plasma concentration-time profile of celecoxib  
182 following oral single dose administration to six horses is graphically presented in Figure 1.  
183 Celecoxib was extensively metabolized after oral administration with low levels of the parent  
184 drug detected in urine for up to 72 hours. The majority of urinary metabolite consisted of  
185 carboxylic acid metabolite which was about 105.53 ng/mL and approximately 3 ng/mL of  
186 hydroxymethyl metabolite of celecoxib at 24 hours after oral administration. Moreover, this  
187 study shows that the carboxylic acid and hydroxymethyl metabolites of celecoxib could be

188 detected in urine for 96 and 48 hours respectively. However, the parent drug and carboxylic  
189 acid metabolite could be detected in faeces for 120 hours after administration (Figure 2).

190 Further, the in vitro study using horse liver microsomes evidenced the formation of  
191 only two metabolites for the parent drug and no other transformations could be detected using  
192 a precursor ion/product ion scan by mass spectrometry (Figure 3).

193

## 194 **Discussion**

195 The use of a sensitive and specific analytical technique for the determination of  
196 celecoxib in biological samples is of paramount importance in pharmacokinetics study. High  
197 performance liquid chromatography with UV or fluorescence detection has been most widely  
198 used in the detection of celecoxib (Rose et al., 2000; Störmer et al., 2003; Zarghi et al., 2006).

199 A liquid chromatography-tandem mass spectrometric quantitation of celecoxib in human  
200 plasma and rat, employing solid phase extraction was developed by (Bräutigam et al., 2001).  
201 Presently mass spectrometry has been predominantly used in the pharmacokinetics and  
202 metabolic study of celecoxib (Zhang et al., 2000; Werner et al., 2002; Ma et al., 2015).

203 The present pharmacokinetic data obtained from horses in this study when compared  
204 to humans and other animals is more or less in complete agreement for celecoxib oral  
205 administration, irrespective of the species differences (Paulson et al., 2000a ; Paulson et al.,  
206 2001; Werner et al., 2002; Itthipanichpong et al., 2005; Park et al., 2012). However, in this  
207 study the plotted plasma concentration-time profile shows that there was a rapid increase in  
208 celecoxib level in about four hours and then the level decreases gradually reaching detectable  
209 levels up to approximately 96 hours following administration (Figure 1). Moreover, the large  
210 volume of distribution ( $V_d/F$ ) and the slow elimination recorded in this study are suggesting  
211 extensive distribution of the drug and / or a poor bioavailability in the body of horses.

212 In the present study, the dosing of 2 mg/kg bwt was based on applying of more or less  
213 the same dose as reported for celecoxib urinary metabolism in horse (Dirikolu et al., 2001; De  
214 Kock et al., 2005), along with the assumption of equal efficacious plasma concentration  
215 between human and horse due to lack of experimental data; and unavailability of celecoxib  
216 pharmaceutical preparation in the veterinary market for horses. However, the found plasma  
217 clearance in this study ( $CL/F = 98.48 \text{ mL/kg/h}$ ) can be of a starting guide to extrapolate a  
218 provisional dose from another species assuming that the same overall body exposure (AUC)  
219 will produce the same effect in both species (Toutain & Bousquet-Mélou, 2004). Considering  
220 the extent of plasma binding is the same between humans and horses with equal  
221 bioavailability factor, F, celecoxib oral dose in horses can be extrapolated tentatively by  
222 applying equation (1); where the reported human plasma clearance ( $CL/F$ ) for 3 mg/kg oral  
223 administration of celecoxib is  $396 \text{ mL/kg/h}$  (Brunton et al., 2018), the calculated dose for  
224 horses is about  $0.75 \text{ mg/kg}$ . Consequently, the average plasma concentration that would be  
225 achieved in steady-state condition in horses with chronic dosing of  $0.75 \text{ mg/kg}$  per day is  
226 about  $317 \text{ ng/mL}$ .

227 Experimental results of this study advise a detection time in horse plasma of  
228 approximately 5 days when celecoxib is administered at  $2\text{mg/kg}$  bwt based on the achieved  
229 analytical LOQ of  $1\text{ng/mL}$ . This also was proven by the generic  
230 pharmacokinetic/pharmacodynamics approach (Toutain & Lassourd, 2002) based on the  
231 determinations of EPC, IPC, SF and the found PK parameters allowing computing of the  
232 detection time (DT) by the equations 2, 3 and 4; where the standard dose in this study is  
233  $2\text{mg/kg}$  and the found plasma clearance is  $98.48\text{mL/kg/h}$ , the calculated EPC is  $846.20\text{ng/mL}$ .  
234 Applying a default safety factor of 500 (Toutain & Lassourd, 2002) will result in irrelevant  
235 plasma concentration of  $1.70\text{ng/mL}$ . Where the slope of the terminal phase ( $\beta$ ) found in the  
236 present study is  $0.05 \text{ h}^{-1}$  and drug maximum concentration in plasma is  $1088 \text{ ng/mL}$ , the

237 computed DT is 5.4 days more or less. Given the above, attention should be given for the  
238 change of detection time with a change in celecoxib dose, typically when applying the  
239 extrapolated therapeutic dose computed with Eqn (1).

240 In humans, celecoxib has been found to be extensively metabolized in the liver with <  
241 3% is excreted unchanged and the major route of elimination are faeces and urine (Davies et  
242 al., 2000). A reported *in vitro* study for celecoxib metabolism using allelic variant forms of  
243 human liver microsomal cytochrome P450 2C9 evidenced the formation of three metabolites,  
244 namely: hydroxylated celecoxib, carboxycelecoxib and its corresponding glucuronide (Tang  
245 et al., 2001). In addition, it was reported that hydroxylation is the primary pathway of  
246 elimination in humans and is similar in several species such as mouse, rat, rabbit, dog and  
247 monkey while the carboxylic acid metabolite of celecoxib undergoes further glucuronidation  
248 to form carboxylic acid glucuronide and is excreted in urine (Paulson et al., 2000b; Paulson et  
249 al., 2001). However, an interesting observation is noticed on the elimination of celecoxib in  
250 horses that the parent drug is excreted as the major component compared to a significantly  
251 lower level of the carboxylic acid metabolite excreted in faeces (Figure 2). The high  
252 concentration of celecoxib in the faeces could be the unabsorbed drug. The two major urinary  
253 metabolites of celecoxib in horses were characterized and identified as 4-hydroxycelecoxib  
254 and 4-carboxycelecoxib (Dirikolu et al., 2001; De Kock et al., 2005). Moreover, it has been  
255 found that there was no appreciable change in the concentration of the carboxylic acid  
256 metabolite when using either hydrolysed or unhydrolysed urine suggesting that the drug or its  
257 metabolite is excreted without conjugation which is evidenced by the absence of any  
258 glucuronic acid conjugate when using a neutral loss screen by LC/MS. Hence, the carboxylic  
259 acid metabolite accounts for more than 90% and the parent drug accounts for 1-2% of the  
260 elimination of celecoxib in horse urine.

261 Rules for controlling medication of animals in competitions are established based on  
262 the possibility to increase artificially both the physical capability and the presence of a  
263 competitive instinct, using drugs. However, an anti-doping policy must not impede the use of  
264 legitimate therapeutic medications and most regulatory bodies in the world now distinguish  
265 the control of illicit substances (doping control) from the control of therapeutic substances  
266 (medication control) (Toutain, 2010). This study indicates that the detection of celecoxib and  
267 its carboxy metabolite are key elements for celecoxib doping investigation in horses, due to  
268 prolonged elimination profile and considerably high detection concentration in urine and  
269 faeces. It is concluded that a detection time of at least 5 days is advised for racing practitioner  
270 and veterinarians after administration of oral therapeutic dose of celecoxib at 2 mg/kg (body  
271 weight) to control its illegitimate use for horse racing. Attention should be given for  
272 computing the detection time upon administration of different oral dose.

273

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276 administration and collection of blood samples.

277

#### 278 **Conflict of interest statement**

279 The authors planned, designed and conducted this study. The authors declare no  
280 conflict of interest.

281

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389 **Table 1**

390 Pharmacokinetic parameters of celecoxib in horses ( $n = 6$ ) after a single oral solution  
391 administration at a dose of 2 mg/kg (body weight). Values are presented as means  $\pm$  standard  
392 deviation (SD).

Pharmacokinetic parameter	Mean $\pm$ SD
Drug maximum concentration in plasma ( $C_{max}$ ) ng/mL	1088 $\pm$ 324
Time to reach peak plasma concentration ( $t_{max}$ ) h	4.58 $\pm$ 1.62
Rate of elimination ( $\beta$ ) $h^{-1}$	0.05 $\pm$ 0.01
elimination half-life ( $t_{1/2\beta}$ ) h	13.60 $\pm$ 3.18
Volume of distribution ( $Vd/F$ ) mL/kg	1904.13 $\pm$ 974.69
Clearance ( $CL/F$ ) mL/h/kg	98.48 $\pm$ 48.16
AUC <sub>0-t</sub> ng.h/mL	24142 $\pm$ 1096

393

394 **Figure legends**

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396 Figure 1: Time course changes (mean  $\pm$  SD) of plasma concentration of celecoxib following  
397 oral administration of a single dose of 2 mg/kg body weight to 6 healthy horses.

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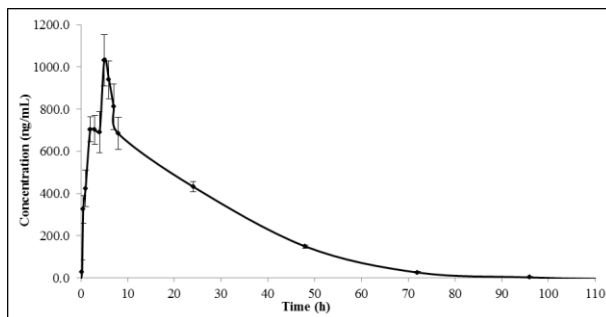
399 Figure 2: Time course changes of faecal excretion of celecoxib and its metabolite COOH-  
400 celecoxib.

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402 Figure 3: LC-MS/MS extracted ion chromatogram and mass spectra of (A) celecoxib (RT = 6.65  
403 min), (B) hydroxymethyl celecoxib metabolite (RT = 6.10 min) and (C) celecoxib carboxylic acid  
404 metabolite (RT = 6.07) obtained from in vitro metabolism of celecoxib using horse liver mircorsomes.

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406 Figure 1:



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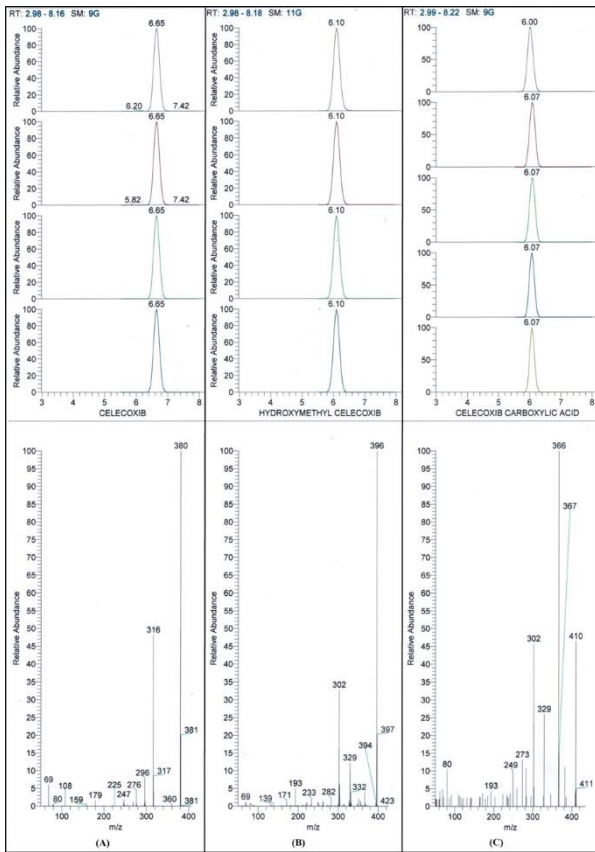
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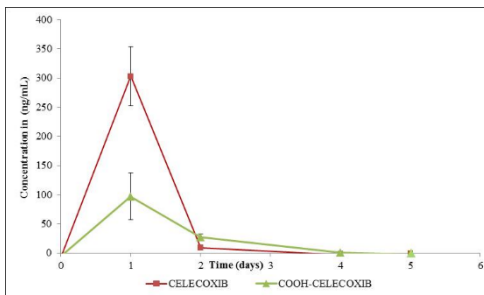
416 Figure 2:



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419 Figure 3:



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