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A clinical evaluation of the pharmacokinetics and pharmacodynamics of intravenous alfaxalone in cyclodextrin in male and female rats following a loading dose and constant rate infusion

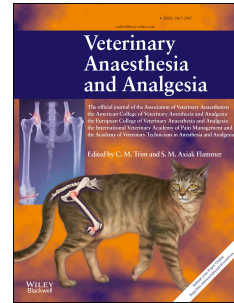
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1 RESEARCH PAPER

2 **A clinical evaluation of the pharmacokinetics and pharmacodynamics of**
3 **intravenous alfaxalone in cyclodextrin in male and female rats following a loading**
4 **dose and constant rate infusion**

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14

15 *Running head: Alfaxalone anaesthesia in male and female rats*

16

17 **Abstract**

18 **Objective** To characterise, as a clinical study, the pharmacokinetics and
19 pharmacodynamics and describe the hypnotic effect of the neurosteroid alfaxalone (3 α -
20 hydroxy-5 α -pregnane-11, 20-dione) formulated with 2-hydroxypropyl- β -cyclodextrin
21 in male and female rats.

22 **Study design** Prospective, experimental laboratory study.

23 **Animals** Twelve (six male and six female) adult, aged matched Sprague Dawley rats.

24 **Methods** Surgery and instrumentation was performed under isoflurane anaesthesia in an
25 oxygen/nitrous oxide mixture (1:2) and local anaesthetic infiltration. All animals
26 received a loading dose (1.67 mg kg⁻¹ minute⁻¹) for 2.5 minutes followed by a constant
27 rate infusion (0.75 mg kg⁻¹ minute⁻¹) for 120 minutes of alfaxalone. Isoflurane and
28 nitrous oxide was discontinued 2.5 minutes after the alfaxalone infusion started.

29 Cardiorespiratory variables (heart rate, respiratory rate, arterial blood pressure, end tidal
30 carbon dioxide tension) and clinical signs of anaesthetic depth were evaluated
31 throughout anaesthesia. Carotid artery blood samples were collected at strategic time
32 points for blood gas analysis, haematology and biochemistry and plasma concentrations
33 of alfaxalone. Plasma samples were assayed using liquid chromatography-mass
34 spectrometry (LC/MS).

35 **Results** There were significant differences between the sexes for plasma clearance ($p =$
36 0.0008), half-life ($p = 0.0268$) and mean residence time ($p = 0.027$). Mean arterial blood
37 pressure was significantly higher in the male rats ($p = 0.0255$).

38 **Conclusions and clinical relevance** This study confirms alfaxalone solubilized in a 2-
39 hydroxypropyl- β -cyclodextrin provides excellent total intravenous anaesthesia in rats.

40 Sex-based differences in pharmacokinetics and pharmacodynamics were demonstrated
41 and must be considered when designing biomedical research models using alfaxalone.

42

43 *Keywords* alfaxalone, anaesthetics, intravenous, rat, steroid,

44

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45 **Introduction**

46 Alfaxalone is a neuroactive steroid that modulates neurotransmission through
47 interaction with a steroid recognition site on the GABA_A receptor complex causing a
48 positive allosteric modulation of the ligand gated chloride channel resulting in
49 inhibition of neuronal excitability (Harrison & Simmonds 1984; Turner et al. 1989).
50 Such agents therefore have roles in anaesthesia, epilepsy, anxiety, insomnia, migraine
51 and drug dependence (Rupprecht & Holsboer 1999). Alfaxalone had been used as an
52 anaesthetic induction agent in humans and veterinary species for almost half a century
53 but anaphylactoid reactions attributed to the polyethoxylated castor oil (Cremophor EL)
54 vehicle (Tammisto et al. 1973) made its use redundant. Subsequent formulations of
55 alfaxalone incorporating a cyclodextrin have hitherto been devoid of the previous side
56 effects and Alfaxan (alfaxalone dissolved in 2-hydroxypropyl- β -cyclodextrin) is now
57 registered for induction and maintenance of anaesthesia in dogs and cats and has been
58 used in horses (Goodwin et al. 2011) sheep (Andaluz et al. 2012; del Mar Granados et
59 al. 2012) rabbits (Navarrete-Calvo et al. 2014) and other more exotic species (Bouts &
60 Karunaratna 2011; McMillan & Leece 2011; Bauquier et al. 2013; Kischinovsky et al.
61 2013; Knotek et al. 2013; Villaverde-Morcillo et al. 2014).

62 The use of alfaxalone in biomedical research and clinical veterinary medicine is gaining
63 popularity as it may offer some selective advantages over other anaesthetic
64 combinations in terms of safety, reflex suppression, cardiopulmonary depression,
65 interaction with receptors involved in pain pathways/modulation and pain on injection
66 (Child et al. 1972; Michou et al. 2012; Santos González et al. 2013) but may also offer
67 additional advantages in influencing CNS development and myelination (Yawno et al.

68 2014). Alfaxalone has been popular for neuroendocrine studies for its sparing of various
69 forebrain functions (Sarkar et al. 1976; Sherwood et al. 1980). Human trials of
70 alphaxalone in cyclodextrin are currently underway (Monagle et al. 2015).
71 The majority of animals used in basic science pain research however are young healthy
72 male laboratory rodents, and indeed it has been suggested that a more heterogeneous
73 and diverse population be used to improve the translational relevance to a human
74 population (Mogil 2009). The inclusion of female rodents is to be encouraged despite
75 the additional complexities that the variability of the oestrous cycle and sexual
76 dimorphism poses; well-designed studies can include both sexes without needless
77 increase in animal numbers (Clayton & Collins 2014). With respect to alfaxalone usage,
78 the pharmacokinetics of a single intravenous (IV) dose have been defined in dogs (Ferré
79 et al. 2006) cats (Whittem et al. 2008b; Muir et al. 2009) female rats (Lau et al. 2013)
80 and male rats after a 5 minute infusion (Visser et al. 2002). The novelty and primary
81 aim of this study was therefore to characterise the pharmacokinetics,
82 pharmacodynamics and hypnotic characteristics of a constant rate infusion of alfaxalone
83 in male *versus* female rats.

84

85 **Materials and methods**

86 This study was performed in accordance with Project Licence PPL30/3156 issued under
87 the Animal (Scientific) Procedures Act 2013 (EU Directive 2010/63/EU) and local
88 ethics committee as part of a larger study investigating nociceptive withdrawal reflexes
89 and diffuse noxious inhibitory control. This study is reported in accordance with the
90 ARRIVE guidelines (Kilkenny et al. 2014).

91 Animals

92 Twelve (9-12 week old) Sprague Dawley rats, six male (397 ± 16 g) and six female
93 (286 ± 20 g) (Charles River Laboratories, Margate, UK) were used. Animals were
94 housed in single sex groups of four, given access to food (Teklad 2018, Harlan) and tap
95 water *ad libitum* and maintained on a 12-hour light/dark cycle. All experiments started
96 at 10:00 h each day.

97

98 General anaesthesia

99 Anaesthesia was induced using 3% isoflurane (Isoflo; Abbott, UK) in oxygen and
100 nitrous oxide mixture (1:2). Once the rat had lost its righting reflex, it was transferred to
101 a heating blanket (Harvard Apparatus Ltd., UK) coupled to a rectal probe for
102 maintenance of body temperature ($37.5 \pm 0.5^\circ\text{C}$). Anaesthesia was maintained using
103 2.00-2.25% (vaporizer setting) isoflurane in oxygen/nitrous oxide delivered via a
104 nosecone. Lidocaine 2% (Lignol; Dechra, UK) 3 mg kg^{-1} was infiltrated subcutaneously
105 prior to skin and sternohyoid incision. The trachea was surgically cannulated using
106 polyethylene 2.42mm O.D. tubing (Fisher Scientific, UK). Respiratory rate and effort
107 was assessed by observing chest excursion and measuring end tidal carbon dioxide
108 (CapStar 100, Linton, Diss, UK). In animals exhibiting respiratory depression as judged
109 by a low respiratory rate and rising end tidal carbon dioxide values, intermittent positive
110 pressure ventilation was initiated (Harvard 683 ventilator, Harvard Apparatus, UK) at
111 60-80 breaths minute^{-1} to maintain end tidal carbon dioxide at 35-45 mmHg (4.67-6.00
112 kPa). The left jugular vein was surgically cannulated using 0.63 mm O.D. polyethylene
113 tubing (Fisher Scientific) for administration of alfaxalone. The left carotid artery was

114 surgically cannulated using 1mm O.D. polyethylene tubing (Fisher Scientific) to
115 monitor arterial blood pressure and for sampling. Arterial blood pressure was monitored
116 by an arterial pressure transducer (Sensonor 840; SensoNor, Norway) and recorded
117 using a PC running Spike2 software (CED Ltd, UK). Heart rate was recorded via two 25
118 gauge needles inserted subcutaneously on the lateral sides of the thoracic wall. The
119 electrocardiogram signal was amplified and used to trigger an instant rate meter
120 (Neurolog NL253, Digitimer, UK) and again recorded using Spike2 software.
121 An infusion of alfaxalone (Alfaxan, Jurox, UK) was started at time 0 at a loading dose
122 of $1.67 \text{ mg kg}^{-1} \text{ minute}^{-1}$ for 2.5 minutes followed by a constant rate infusion (0.75 mg
123 $\text{kg}^{-1} \text{ minute}^{-1}$) for the remainder of the electrophysiological experiment using a
124 calibrated syringe driver (SP100iz, WPI, UK). The isoflurane and nitrous oxide were
125 stopped 2.5 minutes after starting the alfaxalone infusion. Arterial blood was withdrawn
126 from the carotid cannula into lithium heparin and placed on ice. Blood samples (200 μl)
127 were collected at baseline (prior to alfaxalone), end of loading dose, and at 10, 30, 60,
128 90, 120 minutes. Arterial blood gases, biochemistry and haematology parameters (pH,
129 pCO_2 , pO_2 , bicarbonate, sodium, potassium, chloride, calcium, glucose, lactate and
130 creatinine concentrations) were also measured (EPOC, Woodley Instrumentation,
131 Bolton, Lancashire, UK). All rats received an equal volume of balanced electrolyte
132 solution after sampling (Vetivex 11 (Hartmann's); Dechra, UK). Samples were
133 centrifuged (4000g for 10 minutes) within 30 minutes of collection. Plasma was
134 harvested and stored at -20°C until determination of plasma alfaxalone concentration.
135 The hypnotic characteristics of the anaesthetic were evaluated by monitoring paw
136 withdrawal reflex in response to pinch, corneal reflex in response to light brushing,

137 spontaneous blinking and gross purposeful movement and cardiopulmonary parameters.
138 Following the pharmacokinetic study, a separate electrophysiological study was
139 performed, EMG responses were recorded from tibialis anterior, biceps femoris, and
140 medial gastrocnemius muscles during electrical plantar hind paw stimulation of the toes
141 and heel, before and after a conditioning injection of capsaicin into either the
142 contralateral forelimb to study diffuse noxious inhibitory controls (DNIC) or the
143 ipsilateral hind limb to investigate central sensitization and reflex facilitation (Harris &
144 Clarke 2003). Data from this part of the study were not included in this paper but
145 informed the subjective assessment of response to noxious stimuli during alfaxalone
146 anaesthesia. At the end of the experiments animals were euthanised by IV injection of
147 pentobarbitone (Pentobarbital; Ayrton Saunders Ltd, UK) followed by cervical
148 dislocation (as required by UK Home Office regulations). All female rats underwent
149 vaginal swabbing to characterise vaginal smear cell types. Slides were examined under
150 x40 and then x100 magnifications (BH2 microscope, Olympus, UK) after staining with
151 modified Giemsa (Diff Quik, Vet Direct, UK) and cell types and numbers were
152 recorded.

153

154 Sample analyses

155 Samples were analyzed for alfaxalone using a LCMS/MS method. Methanolic standard
156 curve and quality control (QC) spiking solutions were produced for alfaxalone from
157 separate accurate weighings of solid compound. Standards and QCs were prepared by
158 spiking 10 μ l spike solution into a solution of 20 μ l plasma + 30 μ l water + 40 μ l
159 methanol + 150 μ l methanol containing 1000 nM tolbutamide as internal standard.

160 Plasma standard curves were prepared from 100 – 5000 ng mL⁻¹ and QCs were prepared
161 for 250 and 2500 ng mL⁻¹. Blank male or female plasma was used for the standards and
162 QC solutions (Charles River, UK). The plasma samples were prepared by adding 30 µl
163 water + 50 µl methanol + 150 µl methanol containing 1000 nM tolbutamide as internal
164 standard to 20 µl plasma. Samples, standards and QCs were then mixed and stored in a
165 freezer at -20°C for a minimum of 120 minutes prior to centrifugation at 4000g for 20
166 minutes. The samples were extracted and analyzed using a Micromass Quattro Premier
167 mass spectrometer incorporating an Acquity autosampler (Waters, UK). An ACE Excel
168 2 C18-AR 50 x 2.1mm column was used with the following LC conditions: Solvent A =
169 Water + 0.1% Formic Acid, Solvent B = Methanol + 0.1% Formic Acid, Flow rate = 0.8
170 mL minute⁻¹, column temperature = 60°C. LC gradient went from 95 % solvent A:5 %
171 solvent B to 5 % solvent A:95 % solvent B over a 1.5 minute interval. The MS/MS
172 method used electrospray positive mode with a 333.16 >107.01 transition for the
173 detection of alfaxalone. The lower limit of quantification (LLOQ) was 100 ng mL⁻¹. The
174 coefficient of variation at LLOQ was <8% and <16% for other concentration levels. All
175 samples were run in triplicate. Two separate LC/MS/MS runs were performed for the
176 male and female samples, respectively.

177 Samples were analysed within 28 days of collection based on data from analytical
178 validation study file supporting stability of alfaxalone in rat plasma at -20 ° for 30 days
179 (Jurox 2010).

180

181 Pharmacokinetic analyses

182 Pharmacokinetic analysis was carried out using Phoenix WinNonlin 6.3 (Pharsight,
183 Sunnyvale, CA, USA). The pharmacokinetic parameters (clearance, volume of
184 distribution and half life) for each individual rat were estimated according to best fit
185 from an IV infusion one compartmental pharmacokinetic model, based on previous
186 published data (Lau et al. 2013) showing a single exponential decay, with appropriate
187 weighting for best fit. The appropriate weighting for best fit in this study was based on
188 examination of the residuals showing random scatter around predicted values using $1/y^2$
189 weighting by 1/reciprocal of the predicated value.

190

191 Statistical Analyses

192 Statistical tests were performed using GraphPrism (GraphPad Software, CA, USA)
193 version 6. The pharmacokinetic parameters for log transformed parameter data for both
194 genders were compared using an unpaired, two tailed Student's *t*-test and a *p* value of
195 <0.05 was considered significant. Data are reported as mean \pm standard deviation (SD)
196 unless stated otherwise.

197 Cardiopulmonary data were collected continuously, and analyzed at the
198 pharmacokinetic time points. The normality assumptions were tested with Kolmogorov-
199 Smirnov or Shapiro-Wilk tests. The differences in heart rate, mean/systolic/diastolic
200 arterial blood pressure, blood gas variables, lactate, glucose and electrolytes between
201 genders were compared using an unpaired *t*-test. For a more detailed analysis of
202 changes over time, these variables were also analyzed by two-way repeated-
203 measures ANOVA (one factor repetition) for the time points between baseline anaesthesia

204 and 120 minutes after alfaxalone infusion with Sidak's correction for multiple
205 comparisons.

206

207 **Results**

208 Anaesthetic induction and instrumentation were completed without difficulty in all
209 animals. All animals underwent a total of 230 ± 20 minutes of alfaxalone anaesthesia
210 consisting of a 120 minutes of a PK/PD study followed by an electrophysiology study.
211 Only results from the former are reported here.

212

213 Pharmacokinetics

214 The shape of the concentration-time curve following a loading dose and then constant
215 rate infusion was typical of those observed for anaesthetic induction drugs exhibiting an
216 initial steep phase after the loading dose followed by a gradual increase until steady
217 state was achieved. The plasma concentrations were substantially different between the
218 sexes (Figure 1).

219 The pharmacokinetic parameters calculated by an IV infusion one compartmental model
220 are shown in Table 1. Logarithmic transformed data for clearance and $t_{1/2}$ was
221 significantly different between the male and female rats by the two-tailed t -test. As
222 would be expected the MRT was also significantly different between genders.

223

224 Pharmacodynamics

225 Cardiopulmonary data are presented in Table 2.

226 After a 2.5 minute loading dose all rats showed an initial short lived decrease in arterial
227 blood pressure, heart rate and respiratory rate as a result of concomitant administration
228 of inhalant and alfaxalone. Within the next 5 minutes following discontinuation of
229 isoflurane, all rats demonstrated an increase in blood pressure from baseline reading
230 under isoflurane and nitrous oxide anaesthesia. Blood pressure (mean, systolic,
231 diastolic), heart rate and respiratory rate at baseline were not significantly different
232 between male and female rats under isoflurane anaesthesia.

233 Heart rates remained stable during alfaxalone anaesthesia and there was no significant
234 difference between the sexes at any time points.

235 Systolic, mean and diastolic arterial pressures all increased from baseline under
236 isoflurane anaesthesia, reached a peak (between 60 and 90 minutes) and thereafter
237 showed a trend of decreasing with time. Mean arterial blood pressure was significantly
238 different between males and female rats ($p = 0.026$), however the interaction with time
239 ($p < 0.0001$) differed between the genders for mean (and systolic and diastolic) pressure
240 and makes the interpretation of these data difficult (Fig. 2). Significant differences were
241 analyzed *post hoc* using Sidak' multiple comparison test. Mean arterial blood pressure
242 was significantly increased compared to baseline in male rats at 30, 60, 90 and 120
243 minutes from starting the alfaxalone ($p < 0.0005$), as were systolic and diastolic
244 pressures. Mean arterial blood pressure was only significantly greater than baseline in
245 the female rats at 60 minutes. In 4 of 12 animals (2 males and 2 females)
246 cardiopulmonary depression, indicated by a decrease in blood pressure or respiratory
247 rate, necessitated discontinuation of the isoflurane and nitrous oxide before the 2.5

248 minute time point. Within 60 seconds of discontinuation of the isoflurane and nitrous
249 oxide, heart rate and blood pressure began to rise in all animals.

250 All female rats ventilated spontaneously throughout the experiment, whereas 2 of 6
251 male rats required mechanical ventilation as judged by apnoea, or a rise in end tidal
252 carbon dioxide coupled with a decrease in respiratory rate and effort.

253 Blood gas parameters and biochemistry values are presented in Table 3. There were no
254 significant differences between sexes for these parameters except for pH ($p = 0.0027$),
255 which was lower in the female rats in conjunction with higher partial pressures of
256 carbon dioxide. The clinical significance of this is unknown and of little significance.

257 Partial pressures of oxygen were different between baseline and subsequent time points,
258 trending towards higher values under total intravenous anaesthesia compared to
259 inhalational anaesthesia.

260

261 Hypnotic effect

262 The plane of anaesthesia was continually evaluated by serial cardiopulmonary
263 measurements, blood gas analysis and reflex responses. Subjective evaluation of this
264 hypnotic effect of the alfaxalone in all 12 rats was excellent. No rats demonstrated gross
265 purposeful movement or required a change in the infusion rate to improve the plane of
266 anaesthesia.

267

268 Vaginal smears

269 The same investigator read all slides (3 per rat) and evaluated the whole slide to give an
270 impression of the smear, rather than exact cell counts. Three rats were characterized as
271 in dioestrus, one in proestrus, one in oestrus, and one in metoestrus.

272 **Discussion**

273 The major finding from this study is that consideration must be given to the dose of
274 anaesthetic delivered to male and female subjects. Without this interrogation of the
275 experimental model there remains the danger that studies will be carried out under what
276 is assumed to be identical 'planes' of anaesthesia, when in reality one sex may be more
277 or less profoundly anaesthetized such that, for example, hormonal or neuroendocrine
278 responses will be affected.

279 The data reported here were part of an electrophysiological study investigating diffuse
280 noxious inhibitory controls of nociceptive withdrawal reflexes and is part of a larger
281 study of descending control in chronic osteoarthritis, which in humans is more prevalent
282 in females, and highlights the potential risk that using one sex may contribute to the
283 failure of Phase 1 trials or misleading conclusions. In many studies, the influence of the
284 anaesthetic is ignored, or so poorly reported that ensuring a consistent plane of
285 anaesthesia is impossible. Although rats are frequently used in laboratory studies
286 involving anaesthesia, it is typically males used to reduce experimental variability
287 (Zucker & Beery 2010). The limitation of this approach is that basic science intended to
288 be translated into the human population is potentially compromised by the use of one
289 sex (Clayton & Collins 2014). This perceived variability in females is often used as a
290 reason for excluding females from studies. A meta-analysis of 293 studies in which
291 murine behavioural, morphological, physiological, and molecular traits were monitored

292 in both sexes showed variability was not significantly greater in females for any
293 endpoint but several traits contributed to substantially greater variability in the males in
294 this analysis, including the influence of group housing (Prendergast et al. 2014).

295 Commendably, there is a movement towards trying to include more female subjects in
296 studies.

297 The small sample size in our study makes it impossible to postulate that differences
298 within the females are a result of the differences in the oestrous cycle or as a result of
299 normal variability; nonetheless it demonstrates that if steroid hormones can affect
300 alfaxalone efficacy, the stage of the oestrous cycle may also contribute. It is possible
301 that the three female rats requiring additional isoflurane for several minutes between
302 minute 6 and 10 after commencing the alfaxalone may be a result of the stage of the
303 oestrous cycle, but this is impossible to prove definitively.

304 In the research community there remains a collective responsibility for a thoroughness
305 of reporting anaesthesia conditions in order that the anaesthesia is not ‘the elephant in
306 the room’.^a

307

308 Other species e.g. cats display similar pharmacokinetic characteristics to this rat study
309 (Whittem et al. 2008a). The characteristic rapid hepatic metabolic clearance of
310 alfaxalone by the liver has been identified *in-vivo* and *in-vitro* in rats (Sear & McGivan

^a “**Elephant in the room**” is an English metaphorical idiom for an obvious truth that is either being ignored or going unaddressed. The idiomatic expression also applies to an obvious problem or risk no one wants to discuss.

1980; Sear & McGivan 1981). Gender based differences in drug metabolism are the primary cause of sex-dependent pharmacokinetics and reflect differences in the expression of hepatic enzymes active in the metabolism of many extrinsic and intrinsic chemicals, including cytochrome P450 (Waxman & Holloway 2009). Rodent studies have identified more than a 1000 genes whose expression is dependent on sex and these genes modulate liver metabolic function and create sexual dimorphism in liver function (Tanaka 1999). Differences in bioavailability, distribution, metabolism, and/or excretion in different sexes are multifactorial and complicated (Soldin & Mattison 2009). Drug distribution can also be sex linked, influenced by factors such as body fat, plasma volume and differential perfusion of organs. However, in this study no significant difference was recorded between male and female V_{ss} suggesting this was not an issue. In general, however, sex differences in metabolism are thought to be the primary determinant of variation in pharmacokinetics and this is most likely the reason for the differences seen in this study.

325

The quality of anaesthesia was subjectively judged as excellent in all rats. Contrary to previous studies (Brammer et al. 1993), this anaesthetic combination provided very good conditions, stability and survival beyond 180 minutes. The lower blood pressures observed in female rats in this study would suggest that these animals were more profoundly anaesthetized than the males. There was no difference in heart rates between the groups, and the depth of anaesthesia was not so sufficiently profound as to cause apnoea in the females. This is likely to be a pharmacokinetic effect as the females' clearance of alfaxalone is so much less than in the male rats. The consistency of the

334 haematology, biochemistry and blood gas values in all rats demonstrates the stability of
335 the protocol. Blood gas values are infrequently reported for rodent anaesthetics, in part
336 due to the technical nature of artery cannulation, and previously the volume of blood
337 required made repeated sampling impossible due to a deleterious depletion of the blood
338 volume of the animal. Newer point of care analyzers are able to process much smaller
339 volumes. Total blood volume removed was well below the limit of 10% blood volume
340 and this removal of blood in conjunction with a replenishment of balanced electrolyte
341 solution clearly had no impact on the animals. All biochemistry and haematology values
342 except chloride were similar to those provided by the supplier of the Sprague Dawley
343 rats in age-matched subjects. Invariably these samples were analysed with different
344 machines, but even with slight discrepancies usually seen between laboratories, plasma
345 chloride values in the study (mmol L^{-1}) (114 ± 2.6 (females) 115 ± 2.7 (males)) and
346 values in age matched conscious Sprague Dawley rats (109 ± 1.4 (females), 103 ± 1.00
347 (males) were different. A moderate corrected hyperchloraemia was present (when
348 measured sodium values were also taken into consideration). The most likely cause was
349 the administration of normal heparinized saline during cannula placement and through
350 flushing of the carotid cannula with heparinized saline to maintain patency for sampling
351 and blood pressure measurement. A concurrent acidosis was not observed, and sodium
352 values were almost identical between the supplied conscious values and those
353 measured. The potential deleterious effects of normal saline administration have been
354 raised (Handy & Soni 2008) and the administration of non-physiological saline and
355 balanced electrolyte solutions is warranted. The clinical impact of hyperchloraemia is
356 unknown in this study, but in humans there is an increasing awareness that

357 hyperchloraemia and hyperchloraemic acidosis can cause significant clinical
358 ramifications (Handy & Soni 2008).

359

360 Differences in efficacy of the older alfaxalone steroid (Alphathesin/Althesin) in male
361 and female rats has been demonstrated, with males requiring four times the dose of
362 females for surgical anaesthesia and analgesia, and it was concluded that the influence
363 of sex hormones was responsible for this discrepancy (Fink et al. 1982). However a
364 recent study disputed this and postulated that differences are more likely a result of the
365 different formulations of alfaxalone and assay methodologies than differences between
366 sexes (Lau et al. 2013). Notwithstanding these views it has also been shown that
367 formulation is hugely influential; the toxicity of alfaxalone in Wistar rats was less in
368 those animals receiving alfaxalone dissolved in 7-sulfobutyl-ether- β -cyclodextrin
369 (SBECD) compared to alfaxalone in Cremophor EL (Goodchild et al. 2015). The
370 current study directly comparing male and female Sprague Dawley rats receiving an
371 HPCD alfaxan formulation seemingly favours a true sex difference due to
372 pharmacokinetics, pharmacodynamics or both as the explanation. Sex based studies
373 using rats anaesthetized with pentobarbitone have also demonstrated differences
374 (Zambricki & Dalecy 2004). Sex differences have been detected in studies comparing
375 IP and IV routes in rats (Estes et al. 1990) postulating that the lack of obvious sex
376 differences with single IV dosing may be a result of the short duration of effect.

377 Group sizes of 6 were deemed appropriate for evaluating a drug exhibiting within
378 subject variability of less than 30% coefficient of variation of pharmacokinetic
379 measures (Rowland & Tozer 2011) and recent studies comparing cardiovascular effects

380 of anaesthetic drugs have successfully used 5 rats per group (Bencze et al. 2013). It
381 should also be noted that the presence of isoflurane and nitrous oxide at the outset of the
382 loading dose is likely to have affected cardiopulmonary parameters in the very early
383 stages of the infusion and conclusions drawn about differences at these time points are
384 likely to be tenuous. However both groups underwent identical protocols so this
385 influence would have been similar for both groups. The use of an inhalational agent
386 such as isoflurane with minimal metabolism and rapid elimination ensured that the
387 period of time from ceasing administration was as short as possible. The maximum
388 possible duration of initial concurrent administration was 2.5 minutes.

389

390 In summary, there are pharmacokinetic and pharmacodynamic differences with
391 alfaxalone in cyclodextrin in male and female rats. The plane of anaesthesia provided
392 by this protocol is stable and clinically indistinguishable between sexes with no
393 apparent cumulative effect. Half-life, clearance and mean residence time were
394 significantly different between male and female rats indicating that a sex-linked effect
395 was present. The protocol in our study provides excellent anaesthesia conditions but
396 concludes that a dose alteration may be necessary for rat sex-based studies
397 incorporating alfaxalone. This contrasts to previous published studies, which have
398 dismissed a sex difference (Ferré et al. 2006; Berry 2015). Population pharmacokinetics
399 are necessary to further investigate these findings.

400

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404 of interest are declared.

405 **Authors' contributions**

406 KLW, JH: study design and planning; KLW, JH: study conduct; KLW, SWP: data
407 analysis; all authors: paper writing.

408

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549 Figure Legends

550

551 **Figure 1** Mean (\pm SD) alfaxalone plasma concentrations (ng mL^{-1}) for 12 Sprague
552 Dawley rats (6 female, 6 male) after intravenous administration of alfaxalone 1.67 mg
553 $\text{kg}^{-1} \text{ minute}^{-1}$ for 2.5 minutes followed by $0.75 \text{ mg kg}^{-1} \text{ minute}^{-1}$ alfaxalone for 120
554 minutes using a one compartment infusion model.

555

556 **Figure 2** Mean (\pm SD) arterial blood pressure (MAP) (mmHg) for 12 Sprague Dawley
557 rats (6 female, 6 male) after intravenous administration of alfaxalone 1.67 mg kg^{-1}
558 minute^{-1} for 2.5 minutes followed by $0.75 \text{ mg kg}^{-1} \text{ minute}^{-1}$ alfaxalone for 120 minutes
559 using a one compartment infusion model.

560

561

562

563 **Table 1** Pharmacokinetic parameters for 12 Sprague Dawley rats (6 female, 6 male)
 564 after intravenous administration of alfaxalone at a rate of 1.67 mg kg⁻¹ minute⁻¹ for 2.5
 565 minutes followed by 0.75 mg kg⁻¹ minute⁻¹ for 120 minutes using a one compartment
 566 infusion model.
 567

Rat ID	CL (mL minute ⁻¹ kg ⁻¹)	T_{1/2}	Vd_{ss} L kg ⁻¹	MRT minutes	C_{max} mg L ⁻¹
Female 1	66.3	33.8	3.24	48.8	1.3
Female 2	40.4	29.8	1.74	43.0	2.3
Female 3	46.9	76.7	5.19	110.7	0.8
Female 4	58.2	30.9	2.60	44.6	1.6
Female 5	31.9	69.2	3.19	99.9	1.3
Female 6	42.3	34.1	2.08	49.2	2.0
Mean	47.7	45.8	3.00	66.0	1.5
Sd	8.86	20.7	0.6	30.7	0.6
Male 1	65.8	52.1	4.94	75.1	0.8
Male 2	79.9	13.2	1.53	19.1	2.6
Male 3	78.5	17.3	1.95	24.9	2.0
Male 4	117.9	10.3	1.75	14.8	2.2
Male 5	106.2	18.6	2.85	26.8	1.4

Male 6	101.2	26.5	3.87	38.3	1.0
Mean	91.6	23.0	2.82	33.2	1.7
Sd	19.9	15.3	1.36	22.0	0.7
P value	0.0008***	0.0268*	0.710	0.027*	0.780

568

569

570 CL = clearance, $t_{1/2}$ = half life, V_{dss} = volume of distribution, MRT = mean residence
 571 time, C_{max} = maximum plasma concentration

572 CL, $t_{1/2}$ and MRT is significantly different between the male and female rats.

573 Asterisks denote significant difference * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

574

575 **Table 2** Cardiopulmonary parameters for 12 Sprague Dawley rats (6 female, 6 male) after intravenous administration of
 576 alfaxalone at a rate of $1.67 \text{ mg kg}^{-1} \text{ minute}^{-1}$ for 2.5 minutes followed by $0.75 \text{ mg kg}^{-1} \text{ minute}^{-1}$ for 120 minutes using a one
 577 compartment infusion model.

578

Variable	Sex	-5	2.5	10	30	60	90	120
MAP	Male	97 ± 7	71 ± 7	$110 \pm 32^*$	$127 \pm 17^{*****}$	$138 \pm 11^{*****}$	$135 \pm 16^{*****}$	$132 \pm 9^{*****}$
	Female	89 ± 13	90 ± 20	110 ± 17	114 ± 18	$121 \pm 14^{**}$	113 ± 23	114 ± 21
SAP	Male	98 ± 8	96 ± 9	$132 \pm 33^{**}$	$153 \pm 22^{*****}$	$167 \pm 17^{*****}$	$167 \pm 17^{*****}$	$158 \pm 11^{*****}$
	Female	108 ± 12	111 ± 17	127 ± 14	133 ± 15	$144 \pm 15^{**}$	$138 \pm 25^*$	$138 \pm 24^*$
DAP	Male	69 ± 7	60 ± 9	$98 \pm 32^*$	$114 \pm 15^{***}$	$124 \pm 11^{*****}$	$119 \pm 19^{*****}$	$120 \pm 8^{*****}$
	Female	79 ± 14	79 ± 21	101 ± 19	104 ± 19	$109 \pm 14^*$	100 ± 22	101 ± 21
HR	Male	$440 \pm$	$442 \pm$	440 ± 15	455 ± 23	447 ± 28	438 ± 34	423 ± 23

		25	28					
	Female	440 ±	440 ±	436 ± 35	455 ± 23	445 ± 22	415 ± 12	422 ± 16
		31	31					
RR	Male	58 ± 12	55 ± 10	56 ± 10	68 ± 10	57 ± 14	65 ± 7	71 ± 9
	Female	56 ± 10	54 ± 7	56 ± 6	55 ± 5	63 ± 5	68 ± 4	69 ± 4

579 MAP: mean arterial pressure (mmHg); SAP: systolic arterial pressure (mmHg); DAP: diastolic arterial pressure (mmHg); HR:

580 heart rate (beats per minute); RR: respiratory rate (breaths per minute)

581

582 Baseline (-5 minute) samples were all measured under isoflurane (2% vaporizer setting) in N₂O and O₂. Data are mean ± SD.

583 Asterisks denote significant difference from baseline (-5 mins) within a group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

584 using 2 way ANOVA with multiple comparisons.

585

586 **Table 3** Measured blood gas variables and clinical biochemistry parameters for 12 Sprague Dawley rats (6 female, 6 male) after
 587 administration of alfaxalone at a rate of $1.67 \text{ mg kg}^{-1} \text{ min}^{-1}$ for 2.5 minutes followed by $0.75 \text{ mg kg}^{-1} \text{ min}^{-1}$ alfaxalone for 120
 588 minutes using a one compartment infusion model. Data are mean \pm SD.

589

Variable	Sex	Minutes relative to the alfaxalone infusion start time				
		-5	30	60	90	120
pH *	Male	7.36 ± 0.10	7.42 ± 0.12	7.44 ± 0.08	7.41 ± 0.08	7.40 ± 0.06
	Female	7.36 ± 0.14	7.36 ± 0.14	7.35 ± 0.12	7.32 ± 0.10	7.37 ± 0.08
PCO ₂ (mmHg)	Male	36 ± 6	35 ± 12	39 ± 4	40 ± 0.4	30 ± 4
	Female	40 ± 16	44 ± 21	44 ± 16	43 ± 13	37 ± 7
PO ₂ (mmHg)	Male	140 ± 13	218 ± 19	249 ± 13	258 ± 16	276 ± 18
	Female	190 ± 31	318 ± 130	265 ± 54	263 ± 37	219 ± 70
HCO ₃ (mmol L ⁻¹)	Male	23 ± 5	25 ± 2	23 ± 3	23 ± 3	21 ± 3
	Female	24 ± 4	23 ± 4	22 ± 3	21 ± 3	21 ± 9
Sodium (mmol L ⁻¹)	Male	142 ± 2	143 ± 2	142 ± 4	143 ± 2	145 ± 3

	Female	144 ± 6	143 ± 3	147 ± 2	148 ± 5	150 ± 6
Potassium (mmol L ⁻¹)	Male	4.6 ± 0.1	4.8 ± 0.2	4.8 ± 1.1	4.7 ± 0.9	4.5 ± 2.1
	Female	5.2 ± 0.9	4.6 ± 1.2	4.8 ± 0.9	4.7 ± 1.1	5 ± 1.6
Ionized Calcium (mmol L ⁻¹)	Male	1.50 ± 0.1	1.50 ± 0.18	1.47 ± 0.24	1.37 ± 0.34	1.34 ± 0.60
	Female	1.46 ± 0.22	1.50 ± 0.13	1.51 ± 0.19	1.43 ± 0.25	1.37 ± 0.34
Chloride (mmol L ⁻¹)	Male	110 ± 3	116 ± 4	116 ± 6	114 ± 6	117 ± 5
	Female	114 ± 8	110 ± 4	116 ± 4	117 ± 7	114 ± 4
Glucose (mmol L ⁻¹)	Male	12.4 ± 3	7.1 ± 3.6	7.02 ± 3.1	5.8 ± 0.6	5.8 ± 2.8
	Female	13 ± 2.5	7.8 ± 2.5	6.3 ± 3.2	5.4 ± 1	5.9 ± 3.9
Lactate (mmol L ⁻¹)	Male	1.1 ± 0.7	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	0.6 ± 0.1
	Female	1.0 ± 0.1	1.1 ± 0.4	1.0 ± 0.3	1.1 ± 0.6	0.6 ± 0.1
Creatinine (μmol L ⁻¹)	Male	20 ± 7	35 ± 16	28 ± 13	37 ± 13	33 ± 20
	Female	27 ± 12	33 ± 14	29 ± 14	37 ± 19	34 ± 14

590

591

592 Derived variables reported by the EPOC analyzer (actual bicarbonate, total CO₂, base excess of extra cellular fluid, base
593 excess of blood, oxygen saturation, anion gap, anion gap potassium, haemoglobin) were calculated but are not included in the
594 table. The baseline (-5 minute) samples were all measured under isoflurane (2% vaporizer setting) in N₂O and O₂.

595

596 * denotes statistical difference between the groups ($p < 0.05$).

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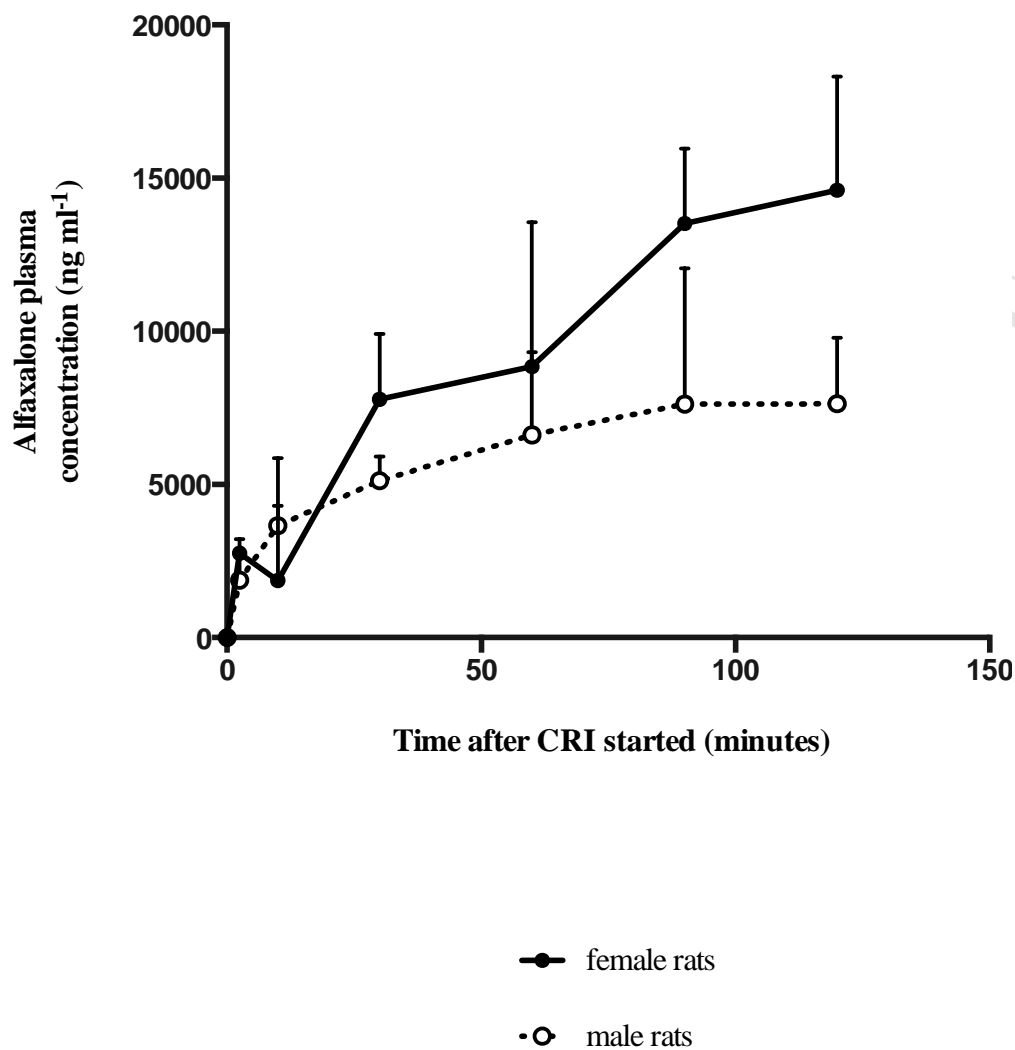


Figure 1 Mean (\pm SD) alfaxalone plasma concentrations (ng mL⁻¹) for 12 Sprague Dawley rats (6 female, 6 male) after intravenous administration of alfaxalone 1.67 mg kg⁻¹ min⁻¹ for 2.5 minutes followed by 0.75 mg kg⁻¹ min⁻¹ alfaxalone for 120 minutes using a one compartment infusion model.

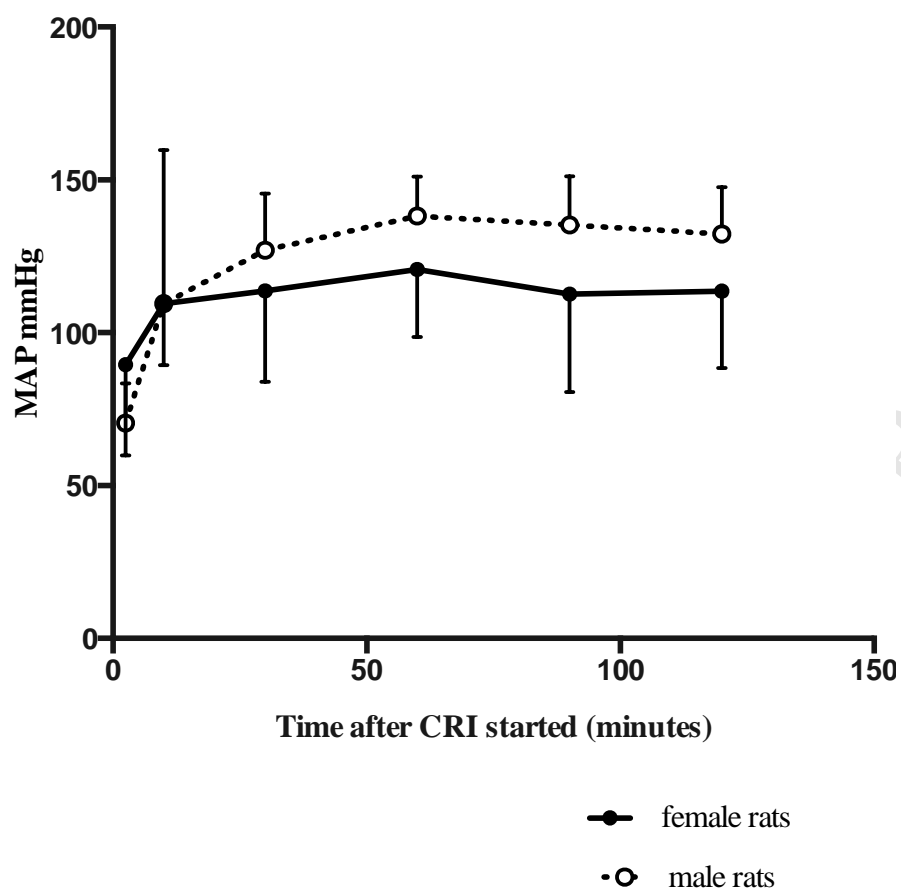


Figure 2 Mean (\pm SD) arterial blood pressure (MAP) (mmHg) for 12 Sprague Dawley rats (6 female, 6 male) after intravenous administration of alfaxalone $1.67 \text{ mg kg}^{-1} \text{ min}^{-1}$ for 2.5 minutes followed by $0.75 \text{ mg kg}^{-1} \text{ min}^{-1}$ alfaxalone for 120 minutes using a one compartment infusion model.