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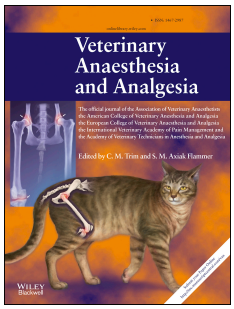

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RESEARCH PAPER

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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Running head: Alfaxalone anaesthesia in male and female rats

Abstract

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

Study design Prospective, experimental laboratory study.

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

Methods Surgery and instrumentation was performed under isoflurane anaesthesia in an oxygen/nitrous oxide mixture (1:2) and local anaesthetic infiltration. All animals received a loading dose (1.67 mg kg $^{-1}$ minute $^{-1}$) for 2.5 minutes followed by a constant rate infusion (0.75 mg kg $^{-1}$ minute $^{-1}$) for 120 minutes of alfaxalone. Isoflurane and nitrous oxide was discontinued 2.5 minutes after the alfaxalone infusion started. Cardiorespiratory variables (heart rate, respiratory rate, arterial blood pressure, end tidal carbon dioxide tension) and clinical signs of anaesthetic depth were evaluated throughout anaesthesia. Carotid artery blood samples were collected at strategic time points for blood gas analysis, haematology and biochemistry and plasma concentrations of alfaxalone. Plasma samples were assayed using liquid chromatography-mass spectrometry (LC/MS).

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

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

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

Keywords alfaxalone, anaesthetics, intravenous, rat, steroid,

Introduction

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

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

2014). Alfaxalone has been popular for neuroendocrine studies for its sparing of various forebrain functions (Sarkar et al. 1976; Sherwood et al. 1980). Human trials of alphaxalone in cyclodextrin are currently underway (Monagle et al. 2015).

The majority of animals used in basic science pain research however are young healthy male laboratory rodents, and indeed it has been suggested that a more heterogeneous and diverse population be used to improve the translational relevance to a human population (Mogil 2009). The inclusion of female rodents is to be encouraged despite the additional complexities that the variability of the oestrous cycle and sexual dimorphism poses; well-designed studies can include both sexes without needless increase in animal numbers (Clayton & Collins 2014). With respect to alfaxalone usage, the pharmacokinetics of a single intravenous (IV) dose have been defined in dogs (Ferré et al. 2006) cats (Whittem et al. 2008b; Muir et al. 2009) female rats (Lau et al. 2013) and male rats after a 5 minute infusion (Visser et al. 2002). The novelty and primary aim of this study was therefore to characterise the pharmacokinetics, pharmacodynamics and hypnotic characteristics of a constant rate infusion of alfaxalone in male *versus* female rats.

Materials and methods

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

Animals

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

General anaesthesia

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

surgically cannulated using 1mm O.D. polyethylene tubing (Fisher Scientific) to monitor arterial blood pressure and for sampling. Arterial blood pressure was monitored by an arterial pressure transducer (SensoNor 840; SensoNor, Norway) and recorded using a PC running Spike2 software (CED Ltd, UK). Heart rate was recorded via two 25 gauge needles inserted subcutaneously on the lateral sides of the thoracic wall. The electrocardiogram signal was amplified and used to trigger an instant rate meter (Neurolog NL253, Digitimer, UK) and again recorded using Spike2 software.

An infusion of alfaxalone (Alfaxan, Jurox, UK) was started at time 0 at a loading dose of 1.67 mg kg^{-1} $minute^{-1}$ for 2.5 minutes followed by a constant rate infusion (0.75 mg kg^{-1} $minute^{-1}$) for the remainder of the electrophysiological experiment using a calibrated syringe driver (SP100iz, WPI, UK). The isoflurane and nitrous oxide were stopped 2.5 minutes after starting the alfaxalone infusion. Arterial blood was withdrawn from the carotid cannula into lithium heparin and placed on ice. Blood samples (200µl) were collected at baseline (prior to alfaxalone), end of loading dose, and at 10, 30, 60, 90, 120 minutes. Arterial blood gases, biochemistry and haematology parameters (pH, pCO_2, pO_2, bicarbonate, sodium, potassium, chloride, calcium, glucose, lactate and creatinine concentrations) were also measured (EPOC, Woodley Instrumentation, Bolton, Lancashire, UK). All rats received an equal volume of balanced electrolyte solution after sampling (Vetivex 11 (Hartmann's); Dechra, UK). Samples were centrifuged (4000*g* for 10 minutes) within 30 minutes of collection. Plasma was harvested and stored at -20°C until determination of plasma alfaxalone concentration.

The hypnotic characteristics of the anaesthetic were evaluated by monitoring paw withdrawal reflex in response to pinch, corneal reflex in response to light brushing,

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

Sample analyses

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

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

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

Pharmacokinetic analyses

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

Statistical Analyses

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

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

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

Results

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

Pharmacokinetics

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

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

Pharmacodynamics

Cardiopulmonary data are presented in Table 2.

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

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

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

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

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

Blood gas parameters and biochemistry values are presented in Table 3. There were no significant differences between sexes for these parameters except for pH ($p = 0.0027$), which was lower in the female rats in conjunction with higher partial pressures of carbon dioxide. The clinical significance of this is unknown and of little significance. Partial pressures of oxygen were different between baseline and subsequent time points, trending towards higher values under total intravenous anaesthesia compared to inhalational anaesthesia.

Hypnotic effect

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

Vaginal smears

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

Discussion

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

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

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

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

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

Differences in efficacy of the older alfaxalone steroid (Alphathesin/Althesin) in male and female rats has been demonstrated, with males requiring four times the dose of females for surgical anaesthesia and analgesia, and it was concluded that the influence of sex hormones was responsible for this discrepancy (Fink et al. 1982). However a recent study disputed this and postulated that differences are more likely a result of the different formulations of alfaxalone and assay methodologies than differences between sexes (Lau et al. 2013). Notwithstanding these views it has also been shown that formulation is hugely influential; the toxicity of alfaxalone in Wistar rats was less in those animals receiving alfaxalone dissolved in 7-sulfobutyl-ether-β-cyclodextrin (SBECD) compared to alfaxalone in Cremophor EL (Goodchild et al. 2015). The current study directly comparing male and female Sprague Dawley rats receiving an HPCD alfaxan formulation seemingly favours a true sex difference due to pharmacokinetics, pharmacodynamics or both as the explanation. Sex based studies using rats anaesthetized with pentobarbitone have also demonstrated differences (Zambricki & Dalecy 2004). Sex differences have been detected in studies comparing IP and IV routes in rats (Estes et al. 1990) postulating that the lack of obvious sex differences with single IV dosing may be a result of the short duration of effect. Group sizes of 6 were deemed appropriate for evaluating a drug exhibiting within subject variability of less than 30% coefficient of variation of pharmacokinetic measures (Rowland & Tozer 2011) and recent studies comparing cardiovascular effects

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

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

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Authors' contributions

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

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549 Figure Legends

550

551 **Figure 1** Mean (± 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 kg^{-1} $minute^{-1}$ for 2.5 minutes followed by 0.75 mg kg^{-1} $minute^{-1}$ alfaxalone for 120
554 minutes using a one compartment infusion model.

555

556 **Figure 2** Mean (± 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 mg kg^{-1} $minute^{-1}$ 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 ½**	**Vdss L kg^{-1}**	**MRT minutes**	**Cmax 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, Vdss = volume of distribution, MRT = mean residence

571 time, Cmax = 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 mg kg $^{-1}$ minute $^{-1}$ for 2.5 minutes followed by 0.75 mg kg $^{-1}$ 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 ± 32*	127± 17****	138 ± 11****	135 ± 16****	132 ± 9****
	Female	89 ± 13	90 ± 20	110 ± 17	114 ± 18	121 ± 14**	113 ± 23	114 ± 21
SAP	Male	98 ± 8	96 ± 9	132 ± 33**	153 ± 22****	167 ± 17****	167 ± 17****	158 ± 11****
	Female	108 ± 12	111 ± 17	127 ± 14	133 ± 15	144 ± 15**	138 ± 25*	138 ± 24*
DAP	Male	69 ± 7	60 ± 9	98 ± 32*	114 ± 15***	124 ± 11****	119 ± 19****	120 ± 8****
	Female	79 ± 14	79 ± 21	101 ± 19	104 ± 19	109 ± 14*	100 ± 22	101 ± 21
HR	Male	440 ±	442 ±	440 ± 15	455 ± 23	447 ± 28	438 ± 34	423 ± 23

		25	28					
	Female	440 ± 31	440 ± 31	436 ± 35	455 ± 23	445 ±22	415 ± 12	422 ± 16
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 N_2O and O_2. 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 mg kg $^{-1}$ min $^{-1}$ for 2.5 minutes followed by 0.75 mg kg $^{-1}$ min $^{-1}$ alfaxalone for 120
588 minutes using a one compartment infusion model. Data are mean ± 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

Derived variables reported by the EPOC analyzer (actual bicarbonate, total CO_2, base excess of extra cellular fluid, base excess of blood, oxygen saturation, anion gap, anion gap potassium, haemoglobin) were calculated but are not included in the table. The baseline (-5 minute) samples were all measured under isoflurane (2% vaporizer setting) in N_2O and O_2.

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

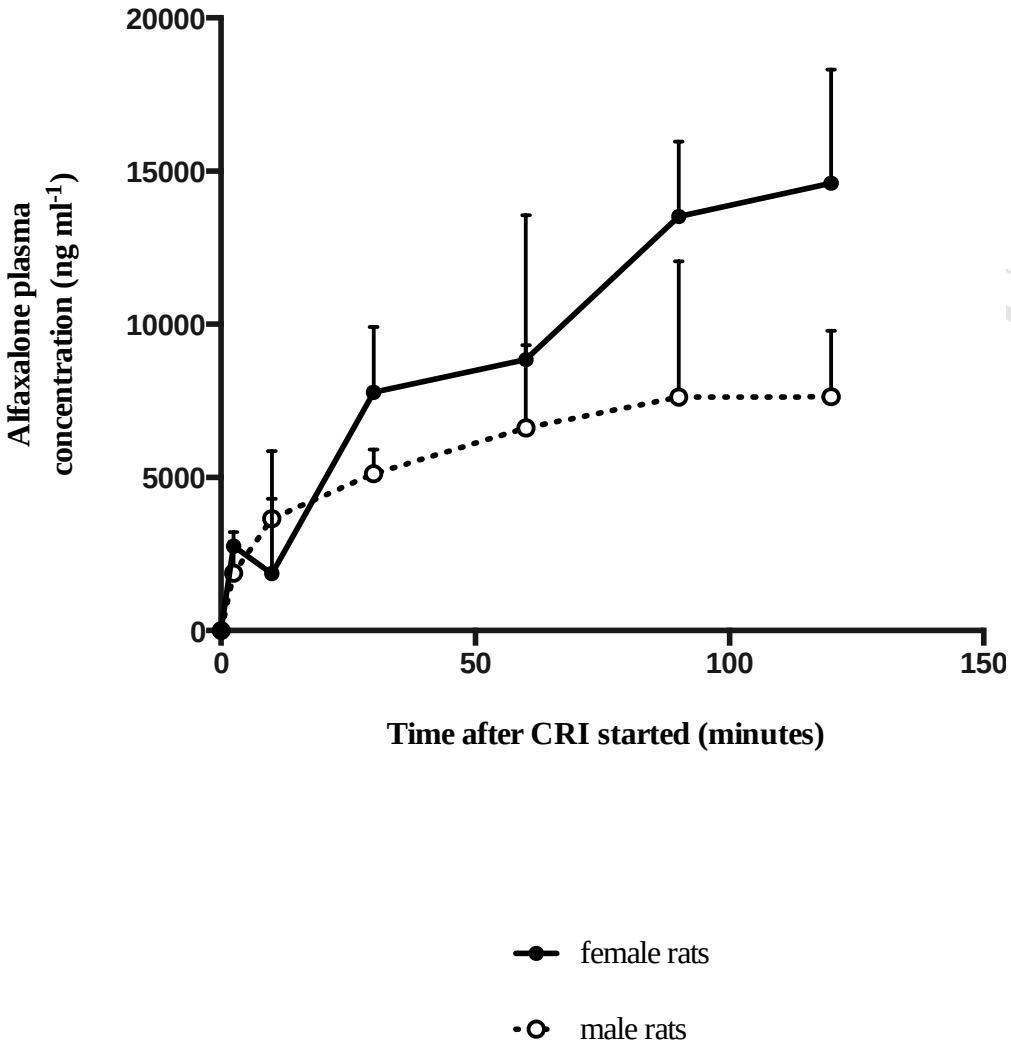

Figure 1 Mean (± SD) alfaxalone plasma concentrations (ng mL^{-1}) for 12 Sprague Dawley rats (6 female, 6 male) after intravenous administration of alfaxalone 1.67 mg kg^{-1} min^{-1} for 2.5 minutes followed by 0.75 mg kg^{-1} min^{-1} alfaxalone for 120 minutes using a one compartment infusion model.

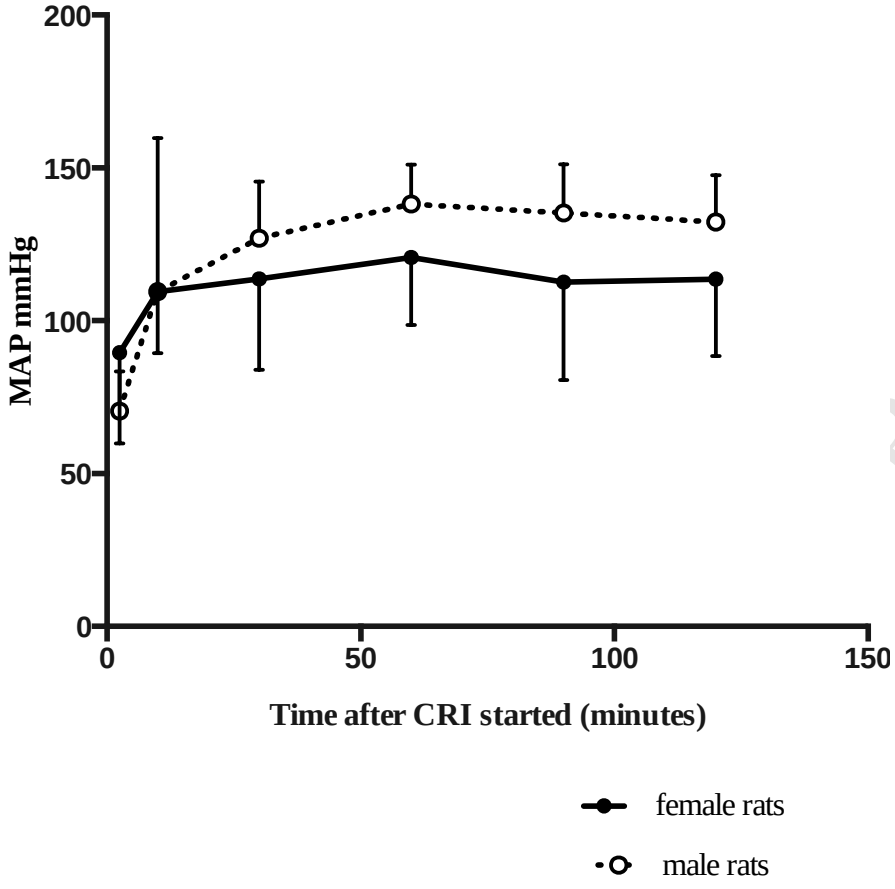

Figure 2 Mean (± SD) arterial blood pressure (MAP) (mmHg) for 12 Sprague Dawley rats (6 female, 6 male) after intravenous administration of alfaxalone 1.67 mg kg $^{-1}$ min $^{-1}$ for 2.5 minutes followed by 0.75 mg kg $^{-1}$ min $^{-1}$ alfaxalone for 120 minutes using a one compartment infusion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