RESEARCH PAPER

Concentrations of vatinoxan and xylazine in plasma, cerebrospinal fluid and brain tissue following intravenous administration in sheep

Magdy Adam[a,b], **Jere Lindén**[c], **Marja Raekallio**[a], **Ahmed Abu-Shahba**[d,e], **Bettina Mannerström**[d], **Riitta Seppänen-Kaijansinkko**[d], **Anna Meller**[f] & **Kati Salla**[a]

[a]Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

[b]Department of Pharmacology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt

[c]Department of Bioscience, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

[d]Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

[e]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Tanta University, Tanta, Egypt

[f]Laboratory Animal Care, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland

Correspondence: Magdy Adam, Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 57, Viikintie 49, 00014, Helsinki, Finland. E-mail: magdy.adam@helsinki.fi

Abstract

Objectives To investigate the extent of vatinoxan distribution into sheep brain, and whether vatinoxan influences brain concentrations of xylazine; and to examine the utility of cerebrospinal fluid (CSF) as a surrogate of brain tissue concentrations for vatinoxan and xylazine.

Study design Randomised, blinded, experimental study.

Animals A total of 14 adult female sheep.

Methods Sheep were randomly allocated into two equal groups and premedicated with either intravenous (IV) vatinoxan (750 μg kg^{-1}, VX) or saline (SX) administered 10 minutes before IV xylazine (500 μg kg^{-1}). Sedation was subjectively assessed at selected intervals before and after treatments. At 10 minutes after xylazine administration, a venous blood sample was collected and the sheep were immediately euthanised with IV pentobarbital (100 mg kg^{-1}). Plasma, CSF and brain tissues were harvested, and concentrations of vatinoxan and xylazine were quantified using liquid chromatography–tandem mass spectrometry. Drug ratios were then calculated and the data were analysed as appropriate.

Results The brain-to-plasma and CSF-to-plasma ratios of vatinoxan were 0.06 ± 0.013 and 0.05 ± 0.01 (mean ± standard deviation), respectively. Xylazine brain concentrations were not significantly different (835 ± 262 *versus* 1029 ± 297 ng g^{-1} in groups VX and SX, respectively) and were approximately 15-fold higher than those in plasma. The CSF-to-brain ratio of vatinoxan was 0.8 ± 0.2, whereas xylazine concentrations in the brain were approximately 17-fold greater than those in CSF, with and without vatinoxan.

Conclusions and clinical relevance Vatinoxan did not significantly affect sedation with xylazine or the concentrations of xylazine in the brain. CSF is not a good predictor of xylazine concentrations in the brain, whereas vatinoxan concentrations were concordant between the brain and CSF, using the dosages in this study.

Keywords brain tissue, cerebrospinal fluid, MK-467, sheep, vatinoxan, xylazine.

Introduction

Simultaneous or prior administration of vatinoxan either attenuates or prevents the adverse cardiopulmonary effects of α_2-adrenoceptor agonists in many animal species, including sheep (Bryant et al. 1998; Raekallio et al. 2010; Adam et al. 2018a,b,c). The level of sedation from dexmedetomidine or medetomidine was not substantially changed by inclusion of vatinoxan in dogs, cats or sheep (Honkavaara et al. 2008, 2017a,b; Raekallio et al. 2010; Restitutti et al. 2011; Adam et al. 2018a,b). In one study in dogs, the plasma medetomidine concentration was markedly decreased by administration of vatinoxan, the duration of sedation was slightly shortened and somatic antinociception was reduced (Bennett et al. 2016). However, the antinociceptive potency of medetomidine was not

significantly affected by vatinoxan when the dosage of medetomidine was adjusted so that the plasma concentrations of medetomidine were equivalent to those produced by medetomidine alone (Huuskonen et al. 2020). Therefore, the minor alterations in the sedative and antinociceptive dose responses induced by vatinoxan may be related to changes in the disposition of the concurrently administered agonist (Vainionpää et al. 2013; Bennett et al. 2016; Honkavaara et al. 2017a,b; Huuskonen et al. 2020). To date, there is no direct evidence for vatinoxan altering the central nervous system (CNS) bioavailability of the co-administered agonists.

Vatinoxan poorly penetrates the blood–brain barrier, thus allowing selective blocking of peripheral α_2-adrenoceptors. Clineschmidt et al. (1988) reported a brain-to-plasma ratio of 0.03 in marmosets and 0.06 in rats following intravenous (IV) administration of vatinoxan (10 mg kg^{-1}). Recently, a comparable brain-to-plasma ratio (0.02) was measured in dogs administered IV vatinoxan (800 μg kg^{-1}) combined with medetomidine (40 μg kg^{-1}) (Honkavaara et al. 2020). In addition, the authors reported a three-to sevenfold brain-to-plasma ratio of medetomidine enantiomers (Honkavaara et al. 2020), comparable to the ratio in rats administered radiolabelled racemic medetomidine (80 μg kg^{-1}) subcutaneously (Salonen 1989) and IV dexmedetomidine (20 μg kg^{-1}) (European Medicines Agency 2005). Furthermore, because of direct contact with the brain tissue, cerebrospinal fluid (CSF) has been used as a common surrogate to measure the unbound brain concentrations of drugs in preclinical and clinical pharmacology studies (Liu et al. 2006; Lin 2008). Currently, no published data exist regarding the CNS distribution of vatinoxan in sheep. Likewise, there are no available data about the feasibility of CSF as a predictor for xylazine and vatinoxan concentrations in brain tissue. Thus, our objectives were to explore the extent of vatinoxan distribution in the sheep brain and study its impact on the central bioavailability of xylazine. Moreover, we aimed to examine the applicability of CSF as a surrogate of brain tissue concentrations. We hypothesised that 1) vatinoxan concentrations in plasma would exceed that in the brain and CSF; and 2) that vatinoxan would not significantly affect the brain-to-plasma concentration ratio of xylazine.

Materials and methods

A group of 14 adult female Texel and cross-bred sheep aged 2.8 ± 0.4 years and weighing 67.8 ± 3.9 kg, means ± standard deviation (SD), were studied. The animals were scheduled for euthanasia for reasons unrelated to this study and the study was approved by the National Animal Experiment Board of Finland (number: ESAVI-16103-2018). The Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines were followed when the study was performed. Sheep were housed indoors as a group and fed hay with free access to water. They were deemed healthy based on clinical examination.

Study protocol

A 16 gauge, 60 mm cannula (Intraflon 2; Vygon, France) was aseptically placed in one jugular vein after subcutaneous infiltration of lidocaine (Lidocaine 2% 0.5 mL; Lidor vet; Richter Pharma AG, Austria) and was used for drug administration. The sheep were randomly (www.randomization.com) assigned to two groups of seven and administered premedication with either vatinoxan (750 μg kg^{-1}; Vetcare Ltd, Finland; group VX) or an equal volume of saline (B. Braun Melsungen AG, Germany; group SX). Vatinoxan was provided as powder and prior to administration was dissolved in isotonic saline (NaCl, 9 mg mL^{-1}; B. Braun Melsungen AG) to a final concentration of 5 mg mL^{-1} and administered as a fast IV bolus over approximately 10 seconds (Time 0 minus 10 minutes; T−10), and the catheter was flushed with 5 mL saline. Xylazine (500 μg kg^{-1}; Nerfasin Vet, 20 mg mL^{-1}; Le Vet Pharma BV, The Netherlands) was injected IV over 5 seconds 10 minutes later (T0), and the catheter was flushed with 5 mL saline. An investigator (AM) who was unaware of group assignment subjectively assessed sedation before drug administration [baseline (BL)], 5 minutes after vatinoxan (time T−5), and 3 and 8 minutes after xylazine (T3 and T8, respectively). Sedation was scored using two scoring systems: a visual analogue score (VAS; a 0–100 mm line marked in 1 mm increments, where 0 denoted no sedation and 100 deep sedation) and a descriptive scale (DS; 0–10, where 0 denoted no sedation and 10 denoted deep sedation with lateral recumbency and no reflexes; Kästner et al. 2003). The elapsed time between xylazine administration and sternal recumbency was recorded.

Blood (6 mL) was collected 10 minutes after xylazine administration (T10) by venipuncture of the jugular vein without a catheter. The blood was placed in ethylenediamine tetraacetic acid tubes (Vacuette tube; Greiner Bio-One GmbH, Austria) centrifuged at 3000 ***g*** for 10 minutes and plasma harvested and stored in duplicate 2 mL cryotubes (Sarstedt AG & Co., Germany) at −20 °C until analysed for drug concentrations. Immediately after blood collection, the sheep were euthanised with IV pentobarbital (100 mg kg^{-1}; Euthanasia solution 400 mg mL^{-1}; Le Vet Pharma BV). After confirming cessation of heartbeats using thoracic auscultation, the sheep were turned into lateral recumbency for CSF collection via the *foramen magnum*. The elapsed time between euthanasia and CSF collection was recorded. A pathologist (JL) performed the dissection following the standard technique for brain removal in small ruminants. The head was separated from the trunk by severing through the atlanto-occipital joint, and the bony roof of cranium (*calvaria*) was exposed by dissecting off the skin. A transversal rostral cut through the forehead and two lateral

diamond-shaped cuts extending to the *foramen magnum* were made and the *calvaria* was removed to expose the brain. Fresh cross-sectional tissue samples of the frontal cortex were harvested and immediately frozen in liquid nitrogen until stored at −80 °C.

Drug concentration analyses

Brain samples (approximately 1 g; frontal cortex) were homogenised with Omni Bead Ruptor (Omni International, GA, USA) in 4× volume (mass per volume) 150 mM phosphate buffer saline (PBS; Admescope, Finland) at 5.65 m second^{-1} for 15 seconds, two cycles, followed by dwell time of 30 seconds. Plasma, brain homogenate and CSF samples were precipitated with 2× volume of acetonitrile (ACN; Fisher Scientific, Finland), including 50 ng mL^{-1} propranolol as internal standards (100 µL sample and 200 µL precipitation solution). Samples were mixed for 3 minutes and centrifuged at 2952 ***g*** for 20 minutes. Precipitated samples were diluted 1:1 with water (100 µL supernatant:100 µL H_2O) and transferred to mass spectrometry analysis. Standards and quality controls were prepared by spiking sheep blank plasma, sheep blank brain homogenate or CSF (Cat# 3525, Batch# 44A; Tocris Bioscience, UK; 10 µL standard/QC and 90 µL blank matrix). Calibrators were prepared at concentrations ranging from 0.2 to 2000 ng mL^{-1}. Quality-control samples were analysed at concentrations of 2.5, 25 and 250 ng mL^{-1}. The matrix specific calibrators and quality-control samples were treated similarly as the samples. Analyses were performed with liquid chromatography−tandem mass spectrometry (Waters Acquity UPLC and Waters TQ-S triple−quadrupole MS; Waters Corp., MA, USA) by use of a reverse-phase C18 column (Waters Acquity BEH C18, 2.1 × 50 mm, 1.7 µm; Waters Corp.). Column temperature was 40 °C, autosampler temperature was 10 °C, and injection volume was 4 µL. Liquid chromatography eluent A was 0.5% formic acid in water, and eluent B acetonitrile. The eluent flow rate was 0.5 mL minute^{-1}. Gradient elution involved changes in solution B as follows: 2% at 0 minutes, 2% at 0.5 minutes, 50% at 2 minutes, 90% at 2.5 minutes, 90% at 3 minutes, and 2% at 4 minutes. Nitrogen was used as desolvation gas (flow rate, 900 L hour^{-1}) and cone gas (flow rate, 150 L hour^{-1}). Solvent temperature was 650 °C, and source temperature was 150 °C. Capillary voltage was 1.0 kV, and ionisation polarity was positive. The samples were analysed in multiple reaction monitoring (MRM) mode using mass-to-change ratio transitions 221 → 105, 419 → 200 and 260 → 116 $m\,z^{-1}$ for xylazine, vatinoxan and internal standard, respectively. The cone voltage was 25 V for xylazine and vatinoxan and 28 V for internal standard. Respective collision energy were 12, 20 and 18 V; and retention times 1.93, 1.81 and 2.38 minutes for xylazine, vatinoxan and internal standard, respectively. Accuracy ranged from 85% to 104% and imprecision values from 2.6% to 6% for all analytes in all matrices. In sheep plasma, the limits of quantitation were 1 and 0.5 ng mL^{-1} for xylazine and vatinoxan, respectively. The corresponding values in sheep brain homogenate and artificial CSF were 0.5 ng mL^{-1} for both analytes.

Statistical analysis

Sample size calculation (http://hedwig.mgh.harvard.edu/sample_size/size.html) indicated that a total of 14 sheep were required if the true difference in brain xylazine concentrations between groups is approximately two times the SD, with 0.9 power and 0.05 alpha level. The plasma, CSF and brain concentrations as well as the calculated brain-to-plasma, CSF-to-plasma and CSF-to-brain ratios were compared between groups with independent-sample *t* test followed by Bonferroni *post hoc* corrections where appropriate. In addition, Spearman's correlations were used to test the associations between plasma, brain and CSF concentrations of vatinoxan and xylazine. Differences in sedation scores were analysed using Mann−Whitney *U* test. The statistical level for significance was set at 0.05. All analyses were computed with IBM SPSS Statistics for Windows, Version 26 (IBM Corp., NY, USA). Data are presented as means ± SD except for sedation scores, which are presented as median (range).

Results

No significant differences were observed between groups in sedation scores (Table 1) or the times to recumbency (4.3 ± 3.5 *versus* 6.8 ± 4.8 minutes for groups VX and SX, respectively).

Prior administration of vatinoxan had no significant effect on xylazine concentrations in plasma or in the brain (Table 2). The time elapsed from euthanasia until CSF sample collection was 8 ± 3 and 9 ± 4 minutes for groups VX and SX, respectively. Vatinoxan concentrations in CSF were comparable to those in the brain, whereas xylazine concentrations in CSF were approximately one-seventeenth of the concentrations in brain (Fig. 1 & Table 2). Two CSF samples, one from each treatment, were excluded because they were apparently contaminated with blood.

Discussion

In the present study, the plasma concentrations of vatinoxan far exceeded the concentrations in brain and CSF. This provides direct evidence of the peripheral selectivity of vatinoxan in sheep. The brain-to-plasma concentration ratio of vatinoxan in sheep was similar to the ratio in rats administered IV vatinoxan (Clineschmidt et al. 1988) and slightly higher than in marmosets (Clineschmidt et al. 1988) and dogs (Honkavaara et al. 2020). Nevertheless, at 20 minutes after administration of a slightly higher dose, vatinoxan concentrations in the brain of

Table 1 Median (range) of visual analogue sedation score (VAS) and descriptive score (DS) in 14 sheep assigned to two equal groups for premedication with either intravenous (IV) vatinoxan (750 μg kg^{-1}, group VX) or an equivalent volume of saline IV (group SX) at time 0−10 minutes, followed 10 minutes later with IV xylazine (500 μg kg^{-1}) at time 0. Sedation was scored before drug administration (baseline; BL), 5 minutes after vatinoxan administration (T−5), and 3 and 8 minutes after xylazine administration (T3 and T8, respectively)

Scoring system	Group	Time points			
		BL	T−5	T3	T8
VAS	VX	0 (0−0)	0 (0−0)	56 (12−72)	65 (27−92)
(0−100 mm)	SX	0 (0−0)	0 (0−0)	44 (22−71)	68 (30−100)
DS (0−10)	VX	0 (0−0)	0 (0−0)	5 (2−7)	7 (3−9)
	SX	0 (0−0)	0 (0−0)	4 (3−7)	7 (4−10)

Table 2 Xylazine and vatinoxan concentrations in plasma, cerebrospinal fluid (CSF) and brain, and the calculated ratios of brain-to-plasma, CSF-to-plasma and CSF-to-brain in sheep premedicated (T−10) with either IV vatinoxan (750 μg kg^{-1}, group VX) or an equivalent volume of saline (group SX), and 10 minutes later (T0) injected IV with xylazine (500 μg kg^{-1}). Data are from seven sheep in each group except for CSF data, which are from six sheep. Data are presented as mean ± standard deviation

Parameter	Xylazine		Vatinoxan
	Group VX	Group SX	Group VX
Plasma (ng mL^{-1})	52 ± 15	68 ± 15	446 ± 57
CSF (ng mL^{-1})	43 ± 13*	61 ± 6	24 ± 6
Brain (ng g^{-1})	835 ± 262	1029 ± 297	27 ± 5
Brain-to-plasma	17 ± 5	15 ± 2	0.06 ± 0.013
CSF-to-plasma	0.90 ± 0.25	1.00 ± 0.31	0.05 ± 0.01
CSF-to-brain	0.06 ± 0.01	0.07 ± 0.02	0.8 ± 0.2

*Significant difference between groups VX and SX ($p < 0.05$).

dogs were comparable to those of the sheep in the present investigation (Honkavaara et al. 2020). These results indicate that vatinoxan has poor penetration of the blood−brain barrier in sheep.

Vatinoxan did not affect the distribution of xylazine in the sheep brain, as no significant difference was observed in the xylazine brain-to-plasma concentration ratio between groups. Therefore, we did not observe changes in the central distribution of the co-administered xylazine by vatinoxan. Correspondingly, no significant difference was detected between groups in xylazine-induced sedation. However, in earlier studies, vatinoxan reduced the concentration of α_2-adrenoceptor agonist (detomidine, medetomidine and dexmedetomidine) in plasma and shortened the duration of sedation in many species when administered IV simultaneously with an α_2-adrenoceptor agonist (Vainionpää et al. 2013; Bennett et al. 2016; Honkavaara et al. 2017a,b; Huuskonen et al. 2020). Under our study setting, sedation was only monitored until euthanasia was performed; therefore, sedation duration was not recorded.

In the present study, the xylazine concentration in CSF was much lower than the concentration in brain. Because of the close relationship between the CSF and brain interstitial fluid, the CSF concentration is frequently used to assess the unbound drug in the CNS and as a surrogate of brain tissue exposure, especially in preclinical studies (Lin 2008). However, this relationship is not straightforward, and for several classes of drugs acting in the CNS, CSF concentrations are either much lower than, or not predictive of, brain tissue concentrations (Liu et al. 2006; Rambeck et al. 2006). For instance, in nonhuman primates, the concentrations of five antiretroviral agents were more than 13-fold lower in CSF than in brain tissue (Srinivas et al. 2019). The study suggested that some of these antivirals might be substrates of certain transporter proteins located in the blood−CSF barrier and effluxes these drugs out from the CSF to blood (Srinivas et al. 2019). Furthermore, the low CSF-to-interstitial fluid ratio of the more lipid-soluble drugs could be attributed to the sink action of CSF, which means that drugs are rapidly cleared by the rapid recycling rate of CSF (Shen et al. 2004). For xylazine, there is a paucity of knowledge on the exact underlying mechanism that dictates its low CSF-to-brain ratio. However, the more likely explanation may be that xylazine was highly bound to brain tissue, which reduced its unbound fraction and consequently resulted in low CSF concentrations. This concept is supported by the so-called 'free drug hypothesis' in pharmacokinetics. This hypothesis states that in the absence of active transportation, the unbound concentration of a drug in blood is equivalent to that in the target tissues at steady state (Shen et al. 2004; Lin 2008; Smith et al. 2010). Recently, Chen et al. (2020) tested this hypothesis in rats and concluded that for drugs with good permeability that are not substrates of efflux transporters, the unbound drug concentration in blood is a reliable surrogate for assessing the unbound concentration in the brain.

Conversely, it was observed in the present study that the CSF and brain concentrations of vatinoxan were comparable. For hydrophilic or large molecular weight compounds with poor to moderate permeability, CSF concentration is a reasonably good discriminating indicator of drug availability to the CNS (Shen et al. 2004). Vatinoxan has a low octanol/phosphate buffer partition coefficient of 1.3 (Clineschmidt et al. 1988) and a relatively large molecular weight of 455 g moL^{-1}. In addition, the presence of an efflux mechanism could be another explanation for the similar CSF-to-brain ratio of vatinoxan. For drugs that are substrates of efflux transporters, the concentrations in the brain and CSF are significantly lower than that in blood (Chen et al. 2020). However, the transcellular movement of vatinoxan has been

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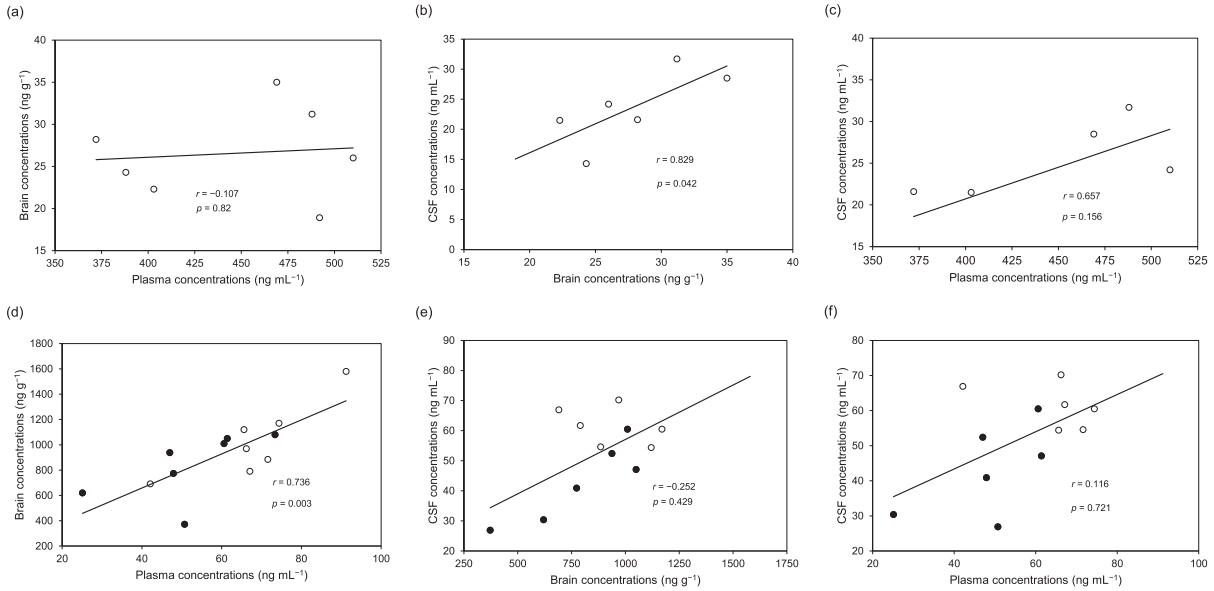

Figure 1 Correlation analyses of vatinoxan and xylazine concentrations in sheep brain, cerebrospinal fluid (CSF) and plasma. Sheep were premedicated with either intravenous (IV) vatinoxan (750 μg kg^{-1}; group VX, $n = 7$) or an equivalent volume of saline (group SX, $n = 7$), followed 10 minutes later with IV xylazine (500 μg kg^{-1}). Correlation analyses of (a) vatinoxan brain concentrations with vatinoxan plasma concentrations; (b,c) vatinoxan CSF concentrations with brain and plasma concentrations; (d) xylazine brain concentrations with xylazine plasma concentrations; and (e,f) xylazine CSF concentrations with brain and plasma concentrations. One CSF sample from each treatment was excluded. Xylazine concentrations were measured in sheep of both groups (VX, black circles; SX, white circles).

assessed in a two-cell line model transfected with human P-glycoprotein (P-gp), and vatinoxan had no apparent permeability in the apical—basolateral direction, suggesting that vatinoxan is not a P-gp substrate (Bennett et al. 2017).

The main limitation of the current study was the lack of vatinoxan treatment alone. Hence, the study design was unable to accurately estimate the extent of distribution of vatinoxan in the brain and whether it was affected by xylazine. Nevertheless, vatinoxan is being considered for clinical use in combination with α_2-adrenoceptor agonists and is unlikely to be used alone. Moreover, owing to the terminal nature of the present experiment, more than one time point for comparisons of brain concentrations was not collected. Thus, this time point was chosen at 20 minutes after vatinoxan injection to relate the concentrations and their ratios to other species from previous studies, namely dogs (Honkavaara et al. 2020), rats and marmosets (Clineschmidt et al. 1988).

Conclusions

The results of this study indicate that vatinoxan has poor penetration to the sheep CNS and has no significant effect on the brain concentration of xylazine. The concentration of xylazine in the CSF was not a good predictor for brain concentrations of xylazine, whereas vatinoxan concentrations were consistent between the brain and CSF, at least with the dosage used in this study. Therefore, a further investigation with various doses of vatinoxan is warranted.

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Authors' contributions

MA and MR: study concept, conduct of experiments and data acquisition, data analysis interpretation, drafting manuscript. JL, AA-S and AM: conduct of experiments and data acquisition. BM and RS-K: planning surgical procedure, acquisition of funding. KS: study concept, conduct of experiments and data acquisition. All authors contributed to critical review and editing of the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest statement

Vetcare Ltd, Finland, provided the vatinoxan used in this study. This company played no role in the study design or in the data acquisition, analysis and interpretation. No author has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. The authors declare no conflict of interest.

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