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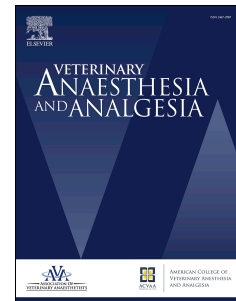
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RESEARCH PAPER

Running head (Authors) *JM Honkavaara et al.*

Running head (short title) Distribution of medetomidine and vatinoxan

Concentrations of medetomidine enantiomers and vatinoxan, an α_2 -adrenoceptor antagonist, in plasma and central nervous tissue after intravenous co-administration in dogs

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Abstract

Objectives To quantify the peripheral selectivity of vatinoxan (L-659,066, MK-467) in dogs by comparing the concentrations of vatinoxan, dexmedetomidine and levomedetomidine in

plasma and central nervous tissue (CNS) after intravenous (IV) co-administration of vatinoxan and medetomidine.

Study design Experimental, observational study.

Animals A group of six healthy, purpose-bred Beagle dogs (four females, two males) aged 6.5 ± 0.1 years (mean \pm standard deviation).

Methods All dogs were administered a combination of medetomidine ($40 \mu\text{g kg}^{-1}$) and vatinoxan ($800 \mu\text{g kg}^{-1}$) as IV bolus. After 20 minutes, the dogs were euthanized with an IV overdose of pentobarbital (140 mg kg^{-1}) and both venous plasma and CNS tissues (brain, cervical and lumbar spinal cord) were harvested. Concentrations of dexmedetomidine, levomedetomidine and vatinoxan in all samples were quantified by liquid chromatography–tandem mass spectrometry and data were analyzed with nonparametric tests with *post hoc* corrections where appropriate.

Results All dogs became deeply sedated after the treatment. The CNS:plasma ratio of vatinoxan concentration was approximately 1:50, whereas the concentrations of dex- and levomedetomidine in the CNS were three to seven-fold those in plasma.

Conclusions and clinical relevance With the doses studied, these results confirm the peripheral selectivity of vatinoxan in dogs, when co-administered IV with medetomidine. Thus, it is likely that vatinoxan preferentially antagonizes α_2 -adrenoceptors outside the CNS.

Keywords central nervous system, distribution, dog, medetomidine, MK-467, vatinoxan.

Introduction

Vatinoxan (formerly known as L-659,066 and MK-467), is an α_2 -adrenoceptor antagonist originally introduced as being peripherally selective as a result of its limited penetration across the mammalian blood-brain-barrier into the central nervous system (CNS) (Clineschmidt et al. 1988). In the last decade, renewed interest in its potential to prevent the peripheral, while preserving the central effects of α_2 -adrenoceptor agonists, has been the subject of numerous studies investigating cardiopulmonary, sedative and metabolic outcomes in several species. In brief, vatinoxan has been shown to either attenuate or prevent the cardiovascular effects of various α_2 -adrenoceptor agonists in dogs (Pagel et al. 1998; Enouri et al. 2008; Honkavaara et al. 2008, 2011; Rolfe et al. 2012), horses (Bryant et al. 1998; de Vries et al. 2016; Tapio et al. 2018), sheep (Bryant et al. 1998; Raekallio et al. 2010; Adam et al. 2018) and cats (Pypendop et al. 2017a; Siao et al. 2017). Moreover, its impact on agonist-induced sedation appears to be minor and more related to alteration on the disposition of co-administered agonists drugs through attenuation of their cardiovascular effects (Vainionpaa et al. 2013; Bennett et al. 2016; Restitutti et al. 2017; Adam et al. 2018; Pypendop et al. 2016, 2017b; Honkavaara et al. 2017a, b). However, to date, direct evidence of the inability of vatinoxan to cross the mammalian blood-brain barrier *in vivo* only exists for rats and marmosets (Clineschmidt et al. 1988). In that study, the concentration of vatinoxan in brain tissue was shown to be a small fraction of that in plasma. On the other hand, the concentration of medetomidine was substantially higher in rat brain than in plasma after subcutaneous (SC) administration (Salonen 1989). To that effect, and as vatinoxan is presently being considered for market authorization in dogs, it would be of importance to confirm its peripheral selectivity in this species. Furthermore, in a recent study by Hector et al. (2017), vatinoxan unexpectedly increased the minimum alveolar concentration (MAC) of sevoflurane in dogs, an effect speculated to be related to possible CNS penetration by the

drug. More recently, similar findings were also reported in cats (Pypendop et al. 2019). As the beneficial effects of α_2 -adrenoceptor agonists, namely sedation, antinociception and anaesthetic-sparing are mediated at the level of the CNS (Doze et al. 1989; Guo et al. 1996; Kita et al. 2000), significant distribution of vatinoxan across the canine blood-brain barrier would potentially decrease its clinical appeal. Hence, we aimed to investigate the extent of its CNS distribution after intravenous (IV) co-administration with medetomidine in dogs. We hypothesized that 20 minutes after treatment administration, the concentration of vatinoxan in plasma would far exceed that of the CNS, whereas the concentrations of medetomidine enantiomers, dex- and levomedetomidine, would be higher in the CNS compared with plasma.

Material and methods

Animals, drugs and study protocol

The study was conducted in accordance with Directive 2010/63/EU and approved by the National Animal Experimentation Board of Finland (permit ID no. ESAVI-2010-04178/Ym23). A group of six research Beagle dogs (four females, two males), scheduled for euthanasia for reasons unrelated to this study, were used. The dogs were considered healthy based on recent clinical and neurological examinations, complete blood counts and serum chemistry. They were housed in a kennel as a single group and fed a commercial diet with free access to water. Mean \pm standard deviation (SD) age and weight were 6.5 ± 0.1 years and 12.5 ± 1.4 kg, respectively. A 20 gauge catheter (Terumo Europe N.V., Belgium) was aseptically placed in the lateral saphenous vein for drug administration. Each dog was administered a combination of medetomidine ($40 \mu\text{g kg}^{-1}$; Dorbene, 1 mg mL^{-1} ; Laboratories Syva, Spain) and vatinoxan ($800 \mu\text{g kg}^{-1}$; Vetcare Ltd, Finland) as a rapid IV bolus followed by 5 mL of 0.9 % sterile saline.

Vatinoxan was obtained as a powder, which was dissolved in sterile isotonic saline to a final concentration of 10 mg mL^{-1} prior to mixing it with medetomidine in a single syringe. The authors have not observed macroscopic physicochemical interactions between the formulations at these concentrations in previous studies. At 20 minutes after treatment administration, 6 mL of blood was collected from the jugular vein. The blood sample was transferred to tubes containing ethylenediamine tetra-acetic acid and pre-chilled in iced water. Plasma was separated by refrigerated centrifugation (4°C , 2520 g for 15 minutes) and stored at -80°C awaiting drug concentration analyses.

Immediately after collecting the blood sample, the dog was euthanized with an overdose of pentobarbital (140 mg kg^{-1} ; Euthasol, 400 mg mL^{-1} ; Le Vet BV, The Netherlands) IV. The CNS was completely removed promptly after confirming cessation of heart beats by thoracic auscultation. Cross-sectional fresh tissue samples of both the lumbar and cervical spinal intumescence and bilaterally of the frontal cortex were immediately frozen in liquid nitrogen and stored at -80°C until analyzed. The dura mater was opened and any remaining spinal fluid removed with an absorbent, non-pilling gauze prior to freezing the spinal samples. Exsanguination was not attempted prior to harvesting the tissues. Macroscopic post-mortem examinations were performed on each dog after the procedures.

Drug concentration analyses

Plasma samples were analyzed for dexmedetomidine, levomedetomidine and vatinoxan concentrations by liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously described in dogs (Honkavaara et al. 2012). For the tissue samples, analytical reference standards for dexmedetomidine and levomedetomidine were purchased from Toronto Research Chemicals (ON, Canada) and vatinoxan was provided by Vetcare Ltd. Calibrator and quality control stock solutions of dexmedetomidine and levomedetomidine

were prepared in methanol and vatinoxan was prepared in 9:1 (v:v) acetonitrile (ACN) and water, all at 1 mg mL⁻¹ free base. The internal standards d4-hydroxydetomidine (d4OHD) and 2-(1-hydroxyethyl) promazine sulfoxide (HEPS) were purchased from Frontier BioPharm (KY, USA) as 0.1 mg mL⁻¹ stocks in methanol. Acetonitrile and water were purchased from Burdick and Jackson (MI, USA). Methanol, methyl tertbutyl ether (MTBE) and buffer reagents were purchased from Fisher Scientific (NJ, USA). Diethylamine (DEA) was from Sigma Aldrich (MO, USA). The solvents were high-performance liquid chromatography grade or better.

For analysis, dexmedetomidine, levomedetomidine and vatinoxan were combined into one working solution. Working solutions were prepared by diluting the 1 mg mL⁻¹ stock solutions with methanol to concentrations of 0.01, 0.1, 1, 10 and 100 ng µL⁻¹. Calibrators were prepared at concentrations ranging from 0.1 ng mL⁻¹ to 2000 ng mL⁻¹. Samples for both calibration curves and negative controls were prepared fresh for each quantitative assay. In addition, quality control samples (at two levels within the standard curve) were included with each set of samples as an additional check of accuracy.

Approximately 230–890 mg of tissue was weighed into 7 mL Precellys hard tissue homogenizing vials (Bertin Corp., MD, USA) and placed in –20 °C for 10 minutes before being homogenized at 510 *g* for 30 seconds in a Precellys 24 tissue homogenizer (Bertin Corp.), followed by a 5-minute cool down period. One mL of ACN containing d4OHD and HEPS at 100 ng mL⁻¹ was added and the samples were homogenized again. The homogenized solution was centrifuged at 15,000 *g* for 2 minutes and 0.5 mL was transferred to a 13×100 mm glass tube and dried under nitrogen at 45 °C.

Samples were re-dissolved with 500 µL of water and 100 µL of 5% ammonium hydroxide in a saturated sodium chloride solution before adding 3 mL of MTBE. Samples were mixed by rotation for 20 minutes at 40 revolutions per minute. After rotation, samples

were centrifuged at 2260 *g* for 5 minutes at 4 °C and the top organic layer was transferred to a glass tube. Samples were then dried under nitrogen at 45 °C and dissolved in 200 μ L of 20 mM ammonium formate with 0.1% DEA. The injection volume into the LC–MS/MS system was 10 μ L.

The concentration of dexmedetomidine, levomedetomidine and vatinoxan were measured in tissue by LC–MS/MS using positive heated electrospray ionization [HESI(+)] at a temperature of 300 °C. Quantitative analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, CA, USA) coupled with a turbulent flow chromatography system (TFC TLX2; Thermo Scientific) having LC-10ADvp liquid chromatography systems (Shimadzu, Japan) and operated in laminar flow mode. The spray voltage was 3500V, the sheath and auxiliary gas were 45 and 30 arbitrary units, respectively. Product masses and collision energies of each analyte were optimized by infusing the analytes into the mass spectrometer. Two injections of each sample were done, one for vatinoxan and another for separation of dexmedetomidine/levomedetomidine. For analysis of vatinoxan, chromatography employed an ACE 3 C18 10 cm \times 2.1 mm 3 μ m column (MAC-MOD Analytical Inc., PA, USA) and a linear gradient of methanol and 20 mM ammonium formate with 0.1% DEA, at a flow rate of 0.35 mL minute⁻¹. The initial methanol concentration was held at 5% for 20 seconds, ramped to 99% over 5 minutes, held at that concentration for 40 seconds, before re-equilibrating at the initial conditions for another 240 seconds. For dexmedetomidine/levomedetomidine analysis, chromatography employed a Lux cellulose 15 cm \times 2.1 mm 3 μ m column (Phenomenex Inc., CA, USA). The isocratic method used the same mobile phases described above at a constant 55% methanol for 6 minutes and 40 seconds.

Detection and quantification were conducted using selective reaction monitoring of initial precursor ion for dexmedetomidine [mass to charge ratio (m/z) 201.1], vatinoxan (m

z^{-1} 419.2), levomedetomidine ($m\ z^{-1}$ 201.1), internal standards d4OHD ($m\ z^{-1}$ 207.1) and HEPS ($m\ z^{-1}$ 345.1). The response for the product ions for dexmedetomidine ($m\ z^{-1}$ 68.2, 95.1), levomedetomidine ($m\ z^{-1}$ 68.2, 95.1), vatinoxan ($m\ z^{-1}$ 199.9, 237.9, 281), d4OHD ($m\ z^{-1}$ 81.1, 189.1), and HEPS ($m\ z^{-1}$ 58.1, 86, 242.9) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). The same software was used to generate calibration curves and quantitate dexmedetomidine, levomedetomidine and vatinoxan in all samples. A weighting factor of $1/X$ was used for all calibration curves.

Quality control samples were included as an additional check of accuracy. Accuracy ranged from 89 to 107 % and imprecision from 3 to 6 % for all analytes. The technique was optimized to provide a limit of quantitation of $0.5\ \text{ng mL}^{-1}$ for both dexmedetomidine and levomedetomidine and $0.1\ \text{ng mL}^{-1}$ for vatinoxan. The limit of detection was approximately $0.2\ \text{ng mL}^{-1}$ for both dexmedetomidine and levomedetomidine and $0.05\ \text{ng mL}^{-1}$ for vatinoxan.

Statistical analysis

Statistical analyses were performed with JMP Pro 12 (SAS Institute, NC, USA). A power analysis was not performed as the number of available animals was dictated by reasons unrelated to this study. Since the number of samples was small, nonparametric tests were used. The calculated tissue:plasma ratios were compared between vatinoxan and dex- and levomedetomidine with Friedman's test, followed by a one-tailed Wilcoxon's Rank Sum test and a Bonferroni *post hoc* correction. The plasma and tissue concentrations between dex- and levomedetomidine were compared by a two-tailed Wilcoxon Rank Sum Test. The alpha-level for significance was set at 0.05. Results are expressed as median (range) or mean \pm SD.

Results

All dogs became profoundly sedated after the treatment and spontaneously remained in lateral recumbency until euthanized. The concentrations for dexmedetomidine, levomedetomidine and vatinoxan and the calculated ratios between plasma *versus* brain and cervical and lumbar spinal cord are summarized in Table 1. The concentration of vatinoxan in the CNS samples was approximately 2% of that measured in plasma, with no significant differences between brain and either of the spinal cord tissues. Whereas the concentrations of dexmedetomidine, levomedetomidine and vatinoxan in the CNS were not significantly different from each other, the CNS:plasma ratios for dex- and levomedetomidine were significantly higher than for vatinoxan ($p < 0.05$). No macroscopic findings indicating disease were present within the CNS or other examined organs in the dogs.

Discussion

This study provided direct evidence of the peripheral selectivity of vatinoxan in dogs, as the concentration in plasma far exceeded concentrations in the CNS tissue. The results for the brain:plasma ratios were similar to those originally reported for marmosets (0.038), when concentrations were measured 20 minutes after IV administration of 10 mg kg^{-1} of vatinoxan (Clineschmidt et al. 1988). In that same study, the brain:plasma ratio for rats administered 57.5 mg kg^{-1} of vatinoxan IV with the same presampling interval was comparable, although consistently somewhat higher (0.06) than in marmosets or the dogs in the present investigation (0.02). Nevertheless, the outcomes of these studies appear remarkably consistent, regardless of species, doses or the fact that the dogs were also administered medetomidine. Consequently, the results in this study indicate that, similarly to rats and marmosets, vatinoxan is peripherally selective in its distribution in dogs. However, the results do not confirm lack of pharmacological action at the level of the CNS. Even though the

concentrations of vatinoxan in the CNS were only a fraction of those in plasma, they were approximately similar to the concentrations of the active dextroisomer in all the sampled tissues. Although it has been reported that plasma concentration ratios (vatinoxan:medetomidine) $\geq 18:1$ were required to effectively attenuate the cardiovascular depression induced by medetomidine in isoflurane-anesthetized dogs (Kaartinen et al. 2014), no such ratio has been suggested for effects in the CNS. As the relative affinity of vatinoxan and dexmedetomidine on α_2 -adrenoceptors is not known, the concentration of vatinoxan needed to relevantly inhibit the pharmacological effects of dexmedetomidine at the receptor site remains to be defined for the relevant α_2 -adrenoceptor subclasses. Consequently, more detailed studies would be required to better define whether effects such as the increase seen in the MAC of sevoflurane in dogs is related to a central or peripheral action (Hector et al. 2017). The study population was homogenous, representing only a small group of healthy dogs of a single breed. Thus, pharmacogenetic variation in, for example, efflux mechanisms across the blood-brain barrier or distribution of α_2 -adrenoceptors may alter the dose-response to vatinoxan.

Vatinoxan increases the volume of distribution of dexmedetomidine in dogs and cats after IV co-administration (Honkavaara et al. 2012; Pypendop et al. 2016). The tissue concentrations in the current study represent only one time point and it is unknown if the concentrations subsequently increased or decreased. However, the exposure to dex- and levomedetomidine within the CNS was three- to seven-fold higher than in plasma at that time. A similar ratio (peak five-fold radioactivity in CNS compared with plasma) was documented in rats 20 minutes after SC administration of $80 \mu\text{g kg}^{-1}$ radiolabeled racemic medetomidine (Salonen 1989). A five to seven-fold radioactivity, brain to plasma, was measured after IV administration of $20 \mu\text{g kg}^{-1}$ radiolabeled dexmedetomidine to rats (European Medicines Agency 2005). In the present study, although concentrations of

vatinoxan and both agonist isomers were higher in the brain than in the spinal cord segments, the differences did not reach statistical significance. However, the brain:plasma concentration ratio of levomedetomidine was significantly higher than that of dexmedetomidine, largely related to the consistent but insignificant lower plasma concentrations of the levoisomer. This phenomenon, which is not easily explained, is in line with a recent study where administration of racemic medetomidine appeared to result in higher relative plasma exposure to dexmedetomidine in dogs (Bennett et al. 2016).

There were some major limitations to this study. First, the study design was unable to include treatments with vatinoxan and medetomidine alone, therefore the results may not accurately describe the distribution of either drug when administered without the other. Nonetheless, it is unlikely that vatinoxan would be administered alone in a clinical scenario. Second, because the methods required invasive tissue sampling, only a single time point was possible for comparisons between tissue and plasma drug concentrations. Therefore, the time of sampling at 20 minutes was chosen to enable comparisons with earlier studies in other species, vatinoxan concentrations in rats and marmosets (Clineschmidt et al. 1988) and medetomidine concentration in rats (Salonen 1989). Furthermore, this time point approximately coincides with the maximal sedative effects of both IV medetomidine and dexmedetomidine in dogs, regardless of the presence of vatinoxan (Honkavaara et al. 2008; Rolfe et al. 2012). Third, the CNS is highly perfused by an extensive transmeningeal microvasculature. Hence, exsanguination prior to tissue collection may have provided more accurate effect-site drug concentrations. By contrast, contamination of the tissue samples with blood would have increased the vatinoxan concentration artificially, falsely increasing the CNS:plasma concentration ratios.

Conclusion

Vatinoxan was shown to be peripherally selective in dogs after IV co-administration with medetomidine. Therefore, the results of this study further support the use of vatinoxan with α_2 -adrenoceptor agonists to attenuate their negative infraspinal effects.

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Authors' contributions

JMH and MRR: study design, data collection, analytical methods and data analysis, preparation of manuscript. PMS: study design, data collection, preparation of manuscript. BHP, HKK and IJK-K: analytical methods and data analysis, preparation of manuscript. OMV: study design, preparation of manuscript.

Conflict of interest

The funding source did not affect planning, data collection, data analysis, conclusions or manuscript writing. The authors declare no conflict of interest.

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Table 1 Concentrations of dexmedetomidine, levomedetomidine and vatinoxan in plasma, brain and cervical and lumbar spinal cord and the calculated concentration ratios for brain:plasma, cervical spine:plasma and lumbar spine:plasma in six dogs 20 minutes after administration of medetomidine ($40 \mu\text{g kg}^{-1}$) and vatinoxan ($800 \mu\text{g kg}^{-1}$) intravenously.

Parameter	Dexmedetomidine	Levomedetomidine	Vatinoxan
Plasma (ng mL^{-1})	6.8 (5.0 – 7.8)	4.1 (3.4 – 5.2)	1380 (701 – 2440)
Brain (ng g^{-1})	29.9 (27.1 – 39.7)	28.2 (22.0 – 37.3)	27.5 (15.6 – 44.3)
Cervical Spine (ng g^{-1})	23.3 (17.8 – 31.2)	18.5 (15.4 – 27.8)	17.2 (12.7 – 20.6)
Lumbar spine (ng g^{-1})	22.0 (14.5 – 31.3)	17.8 (12.9 – 31.0)	20.0 (12.4 – 28.3)
Brain:plasma (ratio)	4.6 (4.3 – 6.9)*	7.2 (5.5 – 7.9)*†	0.02 (0.01 – 0.04)
Cervical spine:plasma (ratio)	3.5 (2.5 – 5.4)*	4.7 (3.5 – 5.4)*	0.01 (0.01 – 0.03)
Lumbar spine:plasma (ratio)	3.4 (2.4 – 4.6)*	4.4 (3.2 – 6.0)*	0.02 (0.01 – 0.02)

Data are expressed as median (range). *Significantly different from vatinoxan ($p < 0.05$).

†Significantly different from dexmedetomidine ($p < 0.05$).