Aus der

Pferdeklinik

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Oral cannabidiol administrations in horses: Pharmacokinetic modelling, behavioural observations and implications for medication control

Inaugural-Dissertation

zur Erlangung des Grades eines

Doctor of Philosophy (PhD) in Biomedical Sciences

an der Freien Universität Berlin

vorgelegt von

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Tierärztin aus Köln

Berlin 2024

Journal-Nr.: 4473

Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus): *horses, cannabidiol, pharmacokinetics, sedatives, behaviour, heart rate, observational studies*

Tag der Promotion: 01.10.2024

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LIST OF ABBREVIATIONS

5-HT_{1A} receptor	Serotonin (5-hydroxytryptamine) 1A receptor
7-COOH-CBD	7-carboxy-CBD
7-OH-CBD	7-hydroxy-CBD
AEA	Anandamide
AST	Aspartate aminotransferase
AUC	Area under the serum concentration curve
BID	Bis in die, *twice daily*
CB_1 receptor	Cannabinoid receptor 1
CB_2 receptor	Cannabinoid receptor 2
CBD	Cannabidiol
Cl	Clearance
C_{max}	Maximum serum concentration
dL	Decilitre
ECS	Endocannabinoid system
EMA	European Medicines Agency
EPC	Effective plasma concentration
F	Bioavailability
FaceSed	Facial sedation scale for horses
FEI	Fédération Équestre Internationale, *International Federation for Equestrian Sports*
FN	Fédération Équestre Nationale, *German Equestrian Federation*
GGT	Gamma-glutamyl transferase
h	Hours
HCPS	Horse Chronic Pain Scale
HGS	Horse Grimace Scale
HR	Heart rate
HRV	Heart rate variability
IPC	Irrelevant plasma concentrations
IU	International unit

IUC	Irrelevant urine concentrations
kg	Kilogram
mg	Milligram
mL	Millilitre
ms	Millisecond
ng	Nanogram
NCA	Non-compartmental analysis
NLME	Nonlinear mixed-effects modelling
NOT	Novel Object Test
p.a.	Post administration
p.o.	Per os, *oral administration*
PK	Pharmacokinetics
Q_2	Intercompartmental clearance between V_1 and V_2
Q_3	Intercompartmental clearance between V_1 and V_3
RR	Reference range
R-R intervals	Cardiac beat-to-beat intervals
RMSSD	Root mean square of successive beat-to-beat differences
Rss	Steady state urine to plasma concentration ratio
SDH	Sorbitol dehydrogenase
SDNN	Standard deviation of normal-to-normal beat-to-beat intervals
SF	Safety factor
SID	Semel in die, *once daily*
$t_{½}$	Terminal half-life
t_{max}	Time of maximum serum concentration
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol
TRPV1 receptor	Transient receptor potential vanilloid 1 receptor
V_1	Volume of distribution in the central compartment
V_2	Volume of distribution in the first peripheral compartment
V_3	Volume of distribution in the second peripheral compartment
Vd	Volume of distribution
Vd_{ss}	Volume of distribution at steady state
WADA	World Anti-Doping Agency

1 INTRODUCTION

The recent legalisations of the plant *Cannabis sativa* for medical purposes in various countries, notably in Germany in 2017, have put *Cannabis* and its numerous compounds, so-called phytocannabinoids, in the spotlight of public interest. The most well-known phytocannabinoids are Δ^9-tetrahydrocannabinol (THC), which produces the typical psychoactive effects associated with cannabis, and cannabidiol (CBD). Products containing CBD extracts have become immensely popular as a supplement for humans and animals: For pets, the global CBD market was estimated at approximately 200 million US-Dollars (USD) in 2022 and is predicted to increase its revenue to 1.71 billion USD in 2030 (Grand View Research 2022).

CBD products are advertised for a broad variety of potential benefits, ranging from general well-being, anxiety and stress relief to anti-inflammatory and analgesic properties, and even support for epileptic patients, all without the psychoactive effects or risk of addiction associated with THC (Corsato Alvarenga et al. 2023; Miranda-Cortés et al. 2022; Potschka et al. 2022; St Blanc et al. 2022). In the animal market, dogs and cats represent the primary target group, yet there's a noticeable trend in creating products tailored specifically to horses such as pastes, pellets or oils. Contrary to the advertised therapeutic benefits of CBD, products for pets are declared as nutritional supplements and are therefore not regulated by the European Medicines Agency (EMA). To date, there is no approved veterinary medicinal CBD product available in the European Union (Briyne et al. 2021).

The rapid expansion of the CBD market continues to widen the gap between scientific evidence and public opinion (Greb and Puschner 2018). Due to the very recent surge of interest, only a few controlled studies in companion animals have been published so far. Investigating the effects of CBD in horses is of particular interest: Given their presumed psychotropic properties, all natural and synthetic cannabinoids are listed as prohibited substances in equestrian sports by the International Federation for Equestrian Sports (FEI) and the German Equestrian Federation (FN). In 2022, the FEI reclassified CBD from “Prohibited Substance - Banned” to “Prohibited substance - Controlled Medication”, thereby recognizing the potential therapeutic value of CBD and the rising prevalence of CBD use in horses (FEI 2022). However, the prohibition of CBD in equestrian sports lacks scientific evidence as very few studies are available regarding detection times and effect of CBD in horses.

The aims of this work were to investigate the pharmacokinetic properties and detection times of CBD following administration of a CBD containing paste in horses. In addition,

behavioural observations and stress tests were conducted to assess the effect of CBD on horses without evident behavioural problems in comparison to a control group.

2 LITERATURE REVIEW – PHARMACOKINETIC ANALYSIS

Pharmacokinetics (PK) describe the absorption, distribution, metabolism and elimination that a drug undergoes within the body. For this purpose, drug concentrations in blood are tracked over time to define PK parameters such as time and amount of maximum concentration, clearance and terminal half-life. Estimation of these parameters can be performed with two approaches: Non-compartmental analysis (NCA) and population pharmacokinetic analysis using statistical modelling (Barnett et al. 2021). NCA derives the desired PK parameters from serum concentration time curves, employing methods like the trapezoidal rule for measurements of the area under the serum concentration curve (Gabrielsson and Weiner 2012). This method is fast and does not require specific assumptions needed for statistical modelling. Population pharmacokinetic analysis aims to derive a more detailed pharmacokinetic assessment of a drug, employing methods such as compartmental modelling. Compartmental modelling divides the body into one to three compartments: A central compartment representing the circulatory system, and up to two peripheral compartments, representing tissues with high and low perfusion (Figure 1). The analysis is performed using methods such as nonlinear mixed-effects modelling (NLME) to describe the pharmacokinetics of a drug specified to these compartments and determine typical PK parameters (Cascone et al. 2013).

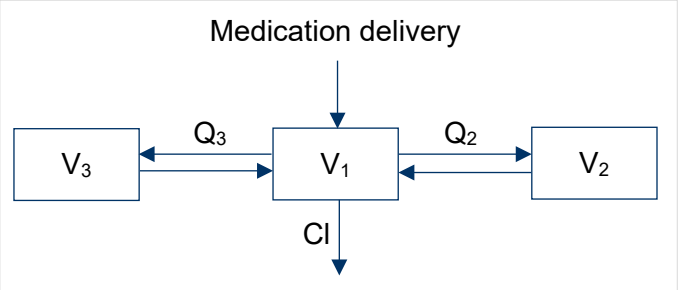

Figure 1: Pharmacokinetic three-compartment model. V_1, volume of distribution in the central compartment; V_2, volume of distribution in the first peripheral compartment; V_3, volume of distribution in the second peripheral compartment; Q_2, intercompartmental clearance between V_1 and V_2; Q_3, intercompartmental clearance between V1 and V3; Cl, total body clearance.

2.1 Pharmacokinetics of CBD in horses

2.1.1 Maximum serum concentrations

Most available equine studies have established CBD pharmacokinetic profiles following oral administrations of 0.35 to 3.0 mg CBD/kg (Draeger et al. 2020; Ryan et al. 2021; Sánchez de Medina et al. 2023; Turner et al. 2022; Williams et al. 2022; Yocom et al. 2022). These dosing amounts are in line with or above the recommendation found on the websites of most

CBD product manufacturers, which is 0.5 mg CBD/kg (Neurogan® 2024; Organica Naturals™ 2024). The maximum CBD concentrations in serum (C_{max}) following single oral administration of 0.5 to 1.0 mg CBD/kg are between 1.7 to 4.3 ng/mL at time points (t_{max}) 1.8 to 4.8 hours (h) post administration (p.a.), see Table 1. Single oral administration of 2.0 to 3.0 mg CBD/kg leads to a higher variety of C_{max}, ranging from 6.1 to 19.9 ng/mL at 2.5 to 5.0 h p.a. (Table 1). One study reported pharmacokinetic parameters following seven days of feeding CBD pellets in doses of (i) 0.35 mg/kg and (ii) 2.0 mg/kg once daily (SID). On the seventh day, comparatively higher CBD serum concentrations were observed: C_{max} was (i) 6.6 ng/mL at t_{max} 1.8 h, and (ii) 51.0 ng/mL at t_{max} 2.4 h p.a. (Williams et al. 2022). The highest single dose tested in horses so far was 10.0 mg CBD/kg (Sánchez de Medina et al. 2023). Here, CBD was administered orally as an oil and a micellar formulation, resulting in a C_{max} of 55.7 ng/mL at 3.5 h and 142.7 ng/mL at 2 h p.a., respectively (Table 1).

CBD pharmacokinetic parameters have also been established in other species following single oral application: In humans, administration of 10 mg CBD (equalling ~ 0.14 mg CBD/kg in a 70 kg human) lead to a C_{max} of 2.5 ng/mL at 1.3 h p.a. (Guy and Robson 2004). Application of higher doses of 400-800 mg (~ 5.8-11.4 mg CBD/kg) reach C_{max} values ranging from 181.2 ng/mL and 221.1 ng/mL, each at 3 h p.a. (Manini et al. 2015). In dogs, oral dosing of 1.0 mg CBD/kg leads to comparatively high C_{max}, ranging from 102 ng/mL to 268 ng/mL at 1.1 to 2.5 h p.a. (Gamble et al. 2018; Tittle et al. 2022; Wakshlag et al. 2020).

Table 1: Overview of single dose oral CBD pharmacokinetic data in horses, sorted by publication date.

Publication	Formulation	Dose (mg/kg)	AUC (ng·h/mL)	C_{max} (ng/mL)	t_{max} (h)	$t_{½}$ (h)
Draeger et al. 2020	Pellets	~ 0.5	-	3.3	2.0	-
Ryan et al. 2021	Oil	0.5	13.2	1.7	2.8	10.7
Ryan et al. 2021	Oil	1.0	23.5	3.2	4.8	10.6
Ryan et al. 2021	Oil	2.0	44.2	6.1	3.2	9.9
Yocom et al. 2022	Lecithin-oil	1.0	73.0	4.3	4.1	14.8
Yocom et al. 2022	Lecithin-oil	3.0	185.7	19.9	5.0	8.5
Turner et al. 2022	Oil	2.0	132.4	18.5	2.5	7.2
Sánchez de Medina et al. 2023	Oil	10.0	778.1	55.7	3.5	34.4
Sánchez de Medina et al. 2023	Micellar	10.0	830.4	142.7	2.0	30.8

AUC, area under the serum concentration curve; CBD, cannabidiol; C_{max}, maximum serum/plasma concentration; $t_{½}$, terminal half-life; t_{max}, time of maximum serum concentration.

2.1.2 Bioavailability

Bioavailability is a relative parameter which describes the availability of a drug for its intended destination (Price and Patel 2023). An intravenously administered drug has a bioavailability (F) of 100%. To obtain the bioavailability of an orally administered drug, the total drug exposure over time is considered, which is expressed by the area under the serum concentration curve (AUC). The respective AUC of an orally administered drug is divided by the AUC of the same drug administered intravenously at the same dose to obtain oral bioavailability (Price and Patel 2023).

Two reports have examined oral and intravenous administration of CBD to determine its oral bioavailability in horses. The findings indicate that, compared to intravenous administration, only 8% to 14% of orally administered CBD is absorbed into the central circulatory system (Sánchez de Medina et al. 2023; Turner et al. 2022). These results are similar to those in humans and dogs, where values for F range between 6% and 19% (Lim et al. 2020; Perucca and Bialer 2020; Samara et al. 1988). Sánchez de Medina et al. (2023) tested bioavailability for oral CBD administered as an oil and a micellar formulation: Despite the vast difference in maximum serum concentrations (Table 1), bioavailability was 14% for both formulations. This is explained by the micellar formulation presenting a faster absorption rate with a steeper slope than the oil formulation, resulting in similar AUC.

The generally low oral bioavailability of CBD is thought to be related to its erratic absorption and extensive first pass effect with considerable pre-systemic hepatic metabolism (Perucca and Bialer 2020; Ryan et al. 2021; Sánchez de Medina et al. 2023).

2.1.3 Distribution

CBD appears to have a high distribution into peripheral tissues. In horses, values for volumes of distribution at steady state (Vd_{ss}) were estimated at 36.0 L/kg following oral (p.o.) administration (Sánchez de Medina et al. 2023), and 5481.7 L/kg following intravenous administration (Turner et al. 2022). Two studies have calculated volumes of distribution with a bias due to unknown bioavailability (F). Yocom et al. (2022) report values for Vd_{ss}/F of 216.7 L/kg and 214.2 L/kg after single administrations of CBD oil in doses 1 mg CBD/kg and 3 mg CBD/kg p.o. Following seven days of feeding CBD pellets in doses 0.35 mg/kg, respectively 2.0 mg/kg SID, Williams et al. (2022) report volumes of distribution at Vd/F: 170 L/kg, respectively Vd/F: 131 L/kg. One study performed population pharmacokinetic analysis and established a three-compartment model (Sánchez de Medina et al. 2023): Values for volumes of distribution were V_1: 0.3 L/kg, V_2: 2.4 L/kg and V_3: 33.3 L/kg. These results are in line with the previously reported tendency of CBD, which is highly lipophilic, to persist in peripheral matrices such as the central nervous system and fatty tissues (Deiana et al. 2012;

Siemens et al. 1980). In other species, Vd_{ss} values were reported at 32.7 L/kg following intravenous administration of 0.3 mg CBD/kg in humans (Ohlsson et al. 1986), and 5.85 L/kg following intravenous administration of 4.5 mg CBD/kg in dogs (Samara et al. 1988).

2.1.4 Metabolism

In humans, CBD is characterised as a drug with a high hepatic clearance and an extraction ratio of 72%, which is the proportion of CBD being eliminated at hepatic first pass (Perucca and Bialer 2020; Wilkinson and Shand 1975). CBD is exclusively metabolised in the liver: The main metabolite generated is 7-hydroxy-CBD (7-OH-CBD), which has the potential to exhibit anticonvulsant effects, and is further metabolised into 7-carboxy-CBD (7-COOH-CBD) (Beers et al. 2021; Kicman and Toczek 2020; Whalley et al. 2017). Little information is available regarding the activity of 7-COOH-CBD and it is commonly regarded as an inactive metabolite (Kicman and Toczek 2020; Whalley et al. 2017). However, in a recent study conducted on human hippocampal neural stem cells, Latham et al. (2023) found that exposure to CBD, 7-OH-CBD as well as 7-COOH-CBD resulted in cell death, particularly evident at higher exposure levels. The concentrations tested were similar to plasma concentrations reported in clinical trials, ranging from 50 ng/mL to 1000 ng/mL, with toxicity increasing in higher doses. Additionally, the study found that high doses of CBD and 7-OH-CBD reduced the percentage of neural stem cells in the first phase of the cell cycle, while 7-COOH-CBD showed no such effect (Latham et al. 2023).

The exact steps of CBD metabolism in horses have not been reported so far, but a certain comparability can be assumed between mammals. One study found that the main metabolites following CBD administration are similar to those in humans, with high concentrations of 7-COOH-CBD in serum and 7-OH-CBD in urine (Ryan et al. 2021). After administration of 1.0 mg CBD/kg p.o., 7-COOH-CBD reached a C_{max} of 85.0 ng/mL at 8.4 h p.a (Ryan et al. 2021). In dogs, the main CBD metabolite is 6-hydroxy-CBD, with only a small amount of 7-OH-CBD identified (Chicoine et al. 2020).

2.1.5 Clearance

One study reported a clearance of 1.46 L/h/kg for CBD in horses (Sánchez de Medina et al. 2023). When set in relation to equine cardiac output (3.38 L/h/kg), the body extraction rate (E) is 0.43, corresponding to CBD having a high blood clearance (Sánchez de Medina et al. 2023; Toutain and Bousquet-Mélou 2004b). Two studies reported clearances biased by an unknown F: Values found for Cl/F were 45.7 L/h/kg and 15.7 L/h/kg, both following single oral administration of 1.0 mg CBD/kg (Ryan et al. 2021; Yocom et al. 2022). In other species, reported clearances were comparatively higher: In humans, CBD clearance was 2546-4741 L/h (~ 36.4-67.7 L/h/kg) following application of 10 mg CBD oromucosal spray (Stott et

al. 2013). In dogs, Cl/F was 9.9 L/h/kg following single oral administration of 10 mg CBD/kg (Doran et al. 2021).

2.1.6 Terminal half-life

Terminal half-life ($t_{½}$) was reported at 7.2-14.8 h following single oral CBD administration in horses (Table 1). After multiple CBD administrations (2.0 mg/kg SID p.o. over 7 days), $t_{½}$ was 13.3 h (Williams et al. 2022). Following single dosing of 10.0 mg CBD/kg p.o., $t_{½}$ in V_1 and V_2 were 0.03 h and 0.75 h. From the third peripheral compartment V_3, $t_{½}$ was 34.4 h (Sánchez de Medina et al. 2023). These results indicate that despite the high blood clearance, the high amounts of CBD accumulation in peripheral tissues lead to an extended elimination process. Terminal half-life is in the same range or lower in other species: Following oral administration of 10-20 mg CBD (~ 0.1-0.3 mg CBD/kg) in humans, $t_{½}$ was reported to be between 1.1-3.0 h (Atsmon et al. 2018a; Atsmon et al. 2018b; Guy and Flint 2004; Guy and Robson 2004). In dogs, $t_{½}$ was 1.0-19.3 h following administration of 1.0-10.0 mg CBD/kg p.o. (Deabold et al. 2019; Di Salvo et al. 2023; Doran et al. 2021).

2.2 Determination of irrelevant drug concentrations for medication control in equestrian sports

Detection of prohibited substances in horses during sport events poses a significant challenge. Commonly used medications have defined cut-off values: Drug concentrations in serum or urine below these values are considered trace and irrelevant for a doping/performance-enhancing effect. To establish these values, Toutain and Lassourd (2002) proposed a pharmacokinetic/pharmacodynamic approach for the assessment of irrelevant drug concentrations in blood and urine samples obtained for medication control during equestrian sports events. This approach requires knowledge of four key variables:

1. Effective standard dose per dosing interval of the drug
2. Bioavailability (F)
3. Plasma clearance (Cl) per dosing interval
4. Steady state urine to plasma concentration ratio (Rss)

These variables are utilized to calculate a drug's effective plasma concentration (EPC) and subsequently derive the irrelevant plasma and urine concentrations (IPC and IUC) of the drug. Calculation of EPC is as follows:

$$\mathrm{EPC} = \frac{\text{Standard dose (per dosing interval) x F}}{\text{Cl (per dosing interval)}}$$

EPC undergoes division by a safety factor (SF), typically established through regulatory decisions (e.g., SF = 500), to determine the irrelevant plasma concentration (IPC):

$$\mathrm{IPC} = \mathrm{EPC} \,/\, \mathrm{SF}$$

IPC is then multiplied by Rss to derive IUC:

$$\mathrm{IUC} = \mathrm{IPC} \times \mathrm{Rss}$$

3 LITERATURE REVIEW – BEHAVIOURAL OBSERVATIONS

3.1 Assessment of stress and anxiety in horses

A variety of options are available for the assessment of stress and anxiety-related behaviour in horses, including behavioural observations and tests as well as the determination of physiological parameters like cortisol levels, heart rate (HR) and heart rate variability (HRV). Purely observational assessments often involve scales for facial expressions. One example is the Horse Grimace Scale (HGS) which aims to assess pain levels in horses at rest through evaluation of facial action units such as orbital tightening, prominent strained chewing muscles and strained nostrils (Dalla Costa et al. 2014). Facial ethograms were also developed for ridden pain assessment (FEReq) with rating of nine facial expressions such as head position and opening of the mouth (Mullard et al. 2017).

Further behavioural tests encompass a horse's response to a stimulus. The Novel Object Test (NOT) is a widely recognized assessment used across species, involving the presentation of an unfamiliar item to an animal (Christensen et al. 2005; Forkman et al. 2007; Munsters et al. 2012; Visser et al. 2002). The test aims to analyse the animals' fear response towards the novel object. Evaluations include behavioural observations and additional parameters like changes in heart rate. Due to the lack of a standardized protocol, the NOT scoring process displays variability across previous reports (Draeger et al. 2021; Forkman et al. 2007; Visser et al. 2002).

Physiological indicators of stress levels include changes in HR and HRV (König von Borstel et al. 2011; Lewinski et al. 2013; Visser et al. 2002; Visser et al. 2003). A decrease in HR alongside an increase in HRV values suggests an autonomic shift towards a parasympathetic dominance, indicating reduced stress levels (Lenoir et al. 2017; Lewinski et al. 2013; Visser et al. 2002). Typical HRV values assessed in horses are the root mean square of successive beat-to-beat differences (RMSSD in milliseconds, ms) and the standard deviation of normal-to-normal beat-to-beat intervals (SDNN, ms) (Visser et al. 2002).

The steroid hormone cortisol is an established physiological stress parameter in horses and can be analysed in blood and saliva (Becker-Birck et al. 2013; Bohák et al. 2013; König v. Borstel et al. 2017; Lewinski et al. 2013; Peeters et al. 2013). Cortisol follows a circadian rhythm with peak levels between 8 am and 12 pm, measuring around 25-70 ng/mL in blood and 0.55-0.70 ng/mL in saliva (Bohák et al. 2013; Irvine and Alexander 1994). Following training or exposure to stress-inducing events, cortisol levels can reach up to 170 ng/mL in blood and up to 7 ng/mL in saliva (Aurich et al. 2015; Bohák et al. 2013; Hall et al. 2014; Malinowski et al. 2006; Peeters et al. 2013).

3.2 Assessment of sedation in horses

Several sedation scores have been proposed for the evaluation of a horse's state after the administration of a sedative agent (Schauvliege et al. 2019). Evaluation of a sedative state can be performed through analysis of facial expressions: The FaceSed incorporates four facial action units, such as the relaxation of the upper and lower lip, to assess a horse's sedative state (Oliveira et al. 2021). More commonly, sedation scores have been developed which rate the horse's head height above the ground or its reaction to an auditory, visual or tactile stimulus (Schauvliege et al. 2019). Poller et al. (2013) employed a sedation scoring system with evaluation of head position, reaction to acoustic stimulation (crackling of a plastic bag) and reaction to visual stimulation (waving of a pink cloth). Each criterion is rated on a scale from 0-3, contributing to a total score classified as no sedation (total score: 0), mild sedation (total score: 1-3), moderate sedation (total score: 4-6) or deep sedation (total score: 7-9).

3.3 Effects of CBD on anxiety, stress and sedation

3.3.1 Molecular targets

The exact pathways of CBD's activity in the body are still under investigation with the most commonly associated target being the endocannabinoid system (ECS). The ECS consists of the G-protein-coupled cannabinoid receptors 1 and 2 (CB_1 and CB_2) and endogenous neurotransmitters, so called "endocannabinoids", as ligands (Almeida and Devi 2020; Bisogno et al. 2001; Russo et al. 2005). The ECS influences synaptic communication and is involved in a number of neurological processes modulating anxiety, eating behaviour, learning, memory, growth and development in humans and animals (Alger and Kim 2011; Devinsky et al. 2014). CB_1 receptors have first been discovered when investigating the target receptors for Δ^9-tetrahydrocannabinol (THC), the phytocannabinoid responsible for the typical psychoactive effects associated with cannabis (Devinsky et al. 2014; Di Marzo and Piscitelli 2015; Pacher et al. 2020; Zou and Kumar 2018). Recent investigations into the binding activity of CBD onto CB_1 and CB_2 receptors found that CBD has very little affinity towards these receptors compared to THC, and even displays antagonistic effects (Almeida and Devi 2020; McPartland et al. 2007; Morales et al. 2017; Pacher et al. 2020; Pertwee et al. 2002; Thomas et al. 2007). CBD does however exhibit an indirect effect on CB_1 and CB_2 receptors by reducing the hydrolysis of the endocannabinoid anandamide (AEA) and consequently allowing an increase in AEA concentrations (Almeida and Devi 2020; Bisogno et al. 2001; Maione et al. 2011). One study reported that regular CBD applications to human patients diagnosed with schizophrenia lead to a significant increase in serum anadamide levels, associated with a clinical improvement (Leweke et al. 2012).

Recent studies identified that the main molecular targets of CBD include serotonin 1A (5-HT_{1A}) and transient receptor potential vanilloid 1 (TRPV1) receptors (Bisogno et al. 2001; Martínez-Aguirre et al. 2020; Morales et al. 2017). Activation of 5-HT_{1A} receptors in humans and laboratory animals has an influence on various physiological and pathological processes including anxiety, mood, depression, immune and cardiovascular regulation (Cowen 2000; Russo 2004; Russo et al. 2005). In human brain tissue, CBD in high concentrations (100 µM) interacts with 5-HT_{1A} receptors in the hippocampus and temporal neocortex (Martínez-Aguirre et al. 2020). CBD was also shown to enhance serotonergic and glutamatergic transmission through modulation of 5-HT_{1A} receptors in the mouse brain, demonstrating an antidepressant-like effect (Linge et al. 2016). Stimulation of TRPV1 receptors leads to vasodilation, inflammation and pain responses, as well as onset and progression of some forms of epilepsy (Anand et al. 2020; Bisogno et al. 2001; Iannotti et al. 2014). CBD was shown to desensitize TRPV1 to nociceptive stimuli and therefore reduce pain and seizure activity (Anand et al. 2020; Bisogno et al. 2001).

3.3.2 Clinical effects on humans

The impact of CBD has been tested in clinical trials on patients with conditions such as epilepsy, chronic pain or anxiety (Peng et al. 2022). Multiple studies showed promising results when CBD was used for the treatment of specific epileptic encephalopathies. However, more research is required to gain a better insight into the general effects of CBD on seizure activity (Wrede et al. 2021). In 2019, EMA has approved the first plant-derived, pharmaceutical-grade CBD medication called Epidyolex® for oral treatment of two epileptic encephalopathies, Lennox-Gastaut Syndrome and Dravet Syndrome (Wrede et al. 2021). Recommended doses typically start at 2.5 mg CBD/kg twice daily and can be increased to a maintenance dose of 10 mg/kg twice daily.

To test CBD as a potential treatment for patients with chronic pain, most studies have explored the effects of an oromucosal spray called Nabiximols (Sativex®) composed of a 1:1 ratio of CBD and THC. The drug is approved for the treatment for spasticity and neuropathic pain in patients with multiple sclerosis (Urits et al. 2020). Dosing is recommended at up to 15 mg (equalling ~ 0.2 mg CBD/kg in a 70 kg human) twice daily. There are limited studies specifically addressing the effectiveness of Nabiximols for treatment of chronic pain, but early results appear promising. However, the efficacy of CBD without THC remains unclear (Urits et al. 2020).

A recently published survey amongst current or past CBD users found that the most frequently named reasons for using non-pharmaceutical CBD are anxiety, sleep problems, stress and general health and well-being (Moltke and Hindocha 2021). More than half of the

questioned users took up to 49 mg CBD (~ 0.7 mg CBD/kg), which is in line with the dosing recommendations stated by most CBD product manufacturers (10-40 mg CBD; ~ 0.1-0.6 mg CBD/kg). These recommendations are however not supported by clinical research: Effective oral doses of CBD for the treatment of mental disorders such as anxiety or social phobia range from 300 to 600 mg (~ 4-9 mg CBD/kg) (Bergamaschi et al. 2011; Crippa et al. 2011; Faria et al. 2020; Zuardi et al. 1993). Regarding the influence CBD has on stress, one study has shown that 600 mg CBD (~ 9 mg/kg) p.o. once daily has the potential to decrease blood cortisol levels (Appiah-Kusi et al. 2020). However, in other reports CBD (600-800 mg p.o. once daily; ~ 9-11 mg CBD/kg) did not have a significant effect on cortisol levels (Hundal et al. 2018; Mongeau-Pérusse et al. 2022). CBD also did not significantly influence responses to negative emotional stimuli and self-reported anxiety following single oral administration of 300-900 mg CBD (~ 4-13 mg CBD/kg) (Arndt and Wit 2017).

At high doses (160-600 mg CBD p.o. once daily; ~ 2-9 mg CBD/kg), CBD has a sedating effect which is reported as either a perceived side effect or as an increase in total sleep time at night (Boggs et al. 2018; Carlini and Cunha 1981; Dos Santos et al. 2021). Another study identified a slight to modest impact of CBD (single dose of 30 mg p.o.; ~ 0.4 mg/kg) on selected resting heart rate variability parameters, but not on HR, RMSSD and SDNN (Williams et al. 2021).

In summary, research on CBD's efficacy for anxiety treatment in humans yields mixed results. The existing evidence suggests that CBD has little impact on physiological stress responses such as blood cortisol levels. Most research involves reactions to acute stressors, with no controlled clinical trials focusing on the effects of repeated CBD administrations to patients with anxiety disorders (Blessing et al. 2015; Sholler et al. 2020).

3.3.3 Clinical effects on horses

In horses, CBD products are advertised for stress reduction and as a potential treatment option for chronic pain and inflammatory disorders. With 0.5 mg/kg, recommended dosing amounts are similar to those in humans. Two studies have tested the effect of CBD as a stress-reducing agent in horses (Draeger et al. 2021; St Blanc et al. 2022). One report performed two novel object tests, one prior to study start and one following six weeks of oral CBD supplementation (~ 0.2 mg/kg SID) (Draeger et al. 2021). Parameters evaluated included heart rate (HR) and reaction scores. When compared to a control group, the HR did not differ, but the reactivity in the CBD group was significantly lower in the second novel object test (Draeger et al. 2021). A second study investigated the effect of CBD on sedation and ataxia using selected grading scales (St Blanc et al. 2022). Horses received an oral CBD supplement

(~ 0.3 mg/kg SID) for eight weeks. No significant differences were identified when compared to a control group (St Blanc et al. 2022).

Case reports on the use of CBD in horses found that a therapy with CBD (0.5 mg/kg twice daily (BID)) led to a significant reduction of oral stereotypic behaviour, namely crib-biting and wind-sucking in a 22-year-old mare (Cunha et al. 2023), and resolution of clinical signs of cutaneous hyperaesthesia and mechanical allodynia in a 4-year-old mare (Ellis and Contino 2021).

A recent study has assessed the effect of orally administered CBD over 14 days on the presence of pain in osteoarthritic patients (Interlandi et al. 2024). Horses were divided into two groups, one group treated with phenylbutazone and hemp oil (0.03 mg CBD/kg SID p.o.), and a control group treated with phenylbutazone alone. Pain levels were assessed daily using the Horse Chronic Pain Scale (HCPS; van Loon and Macri 2021). On days 9-14, horses in the CBD group scored significantly lower on the HCPS, indicating a reduced pain expression compared to horses in the control group. Additionally, physiological parameters such as heart rate, respiratory rate, white blood cell count and malondialdehyde - a biomarker of oxidative stress - were significantly lower on days 12-14 in the CBD than in the control group (Interlandi et al. 2024).

3.3.4 Clinical effects on other species

CBD use in dogs is mostly associated with the treatment of neurological conditions such as epilepsy (Williamson et al. 2021) and pain management in chronic diseases like osteoarthritis (Mosley et al. 2021). Epilepsy is one of the most prevalent neurological disorders in dogs and there is a growing interest in exploring the effectiveness of CBD for its potential antiseizure effects (Kogan et al. 2018; Lacombe et al. 2012; Potschka et al. 2022). CBD as a treatment for epilepsy was tested in two studies which showed a reduction in seizures of up to 42% following oral treatment (2.0-2.5 mg CBD/kg BID) over twelve weeks (Garcia et al. 2022; McGrath et al. 2019). In contrast, no consistent effects were found in a case report of three dogs with suspected epilepsy (Mogi and Fukuyama 2019). Studies performed in dogs with osteoarthritis identified a significant reduction of pain and increased activity following administration of up to 4 mg CBD/kg BID for twelve weeks (Di Salvo et al. 2023; Kogan et al. 2020).

There are few reports on the impact of CBD on stress and anxiety in dogs: One study investigated the effect CBD has on dog behaviour, focusing on signs of stress and aggression (Corsetti et al. 2021). Following oral administration of CBD (1 drop of 5% CBD oil/2 kg; equalling ~ 0.00125 mg CBD/kg SID for 45 days), no significant effect was found regarding stress related behavioural patterns, the dogs' level of attention and the perception of the

environment and surrounding stimuli. Aggressive behaviour towards humans was shown to have decreased significantly over time. However, no significant difference in the decrease of aggressive behaviour towards humans was seen when compared to a control group (Corsetti et al. 2021).

Another report tested the effect of CBD (up to 4.5 mg/kg SID p.o. for 21 days) on activity levels in healthy dogs and found no significant alteration in daily activity or quality of sleep (Morris et al. 2021). One study investigated the effectiveness of a single CBD administration (4.0 mg/kg) on blood cortisol levels, HR and HRV during a stress test (Hunt et al. 2023). Dogs were rated as significantly less stressed and had significantly lower cortisol levels compared to a control group. HR and HRV did not differ between groups (Hunt et al. 2023). Similarly, dogs receiving 1.4 mg CBD/kg SID p.o. for 31 days showed no significant changes in RMSSD and SDNN following a fear response test (Morris et al. 2020). In surveys among US veterinarians and dog owners, sedation was frequently named as a perceived side effect following administration of CBD (Kogan et al. 2016; Kogan et al. 2018; Kogan et al. 2019).

In rats, CBD (10 mg/kg, single intraperitoneal injection) significantly reduced the increase of HR and blood pressure in a stress inducing and fear conditioning setting, suggesting an anxiolytic effect similar to the effect of diazepam (Resstel et al. 2006; Resstel et al. 2009).

3.4 Safety and side effects

In horses, Yocom et al. (2022) reported mildly elevated fibrinogen levels (500-600 mg/dL, reference range (RR): 100-400 mg/dL) and liver enzymes (Gamma-glutamyl transferase (GGT): 28-98 IU/L, RR: 10-25 IU/L; Aspartate aminotransferase (AST): 385-838 IU/L, RR: 185-375 IU/L; Sorbitol dehydrogenase (SDH): 11-30 IU/L, RR: 0-10 IU/L), as well as mild hypocalcemia (10.0–11.4 mg/dL; RR: 11.5–14.0 mg/dL) following single oral CBD administration in doses 1 mg/kg and 3 mg/kg. Values returned to reference ranges following discontinuation of CBD application. Physical examinations remained within normal limits throughout the duration of the study (Yocom et al. 2022). Another report found a significant increase in albumin levels from 3.0 ± 0.2 g/dL at baseline to 3.5 ± 0.1 g/dL following 90 days of daily CBD administrations (2 mg CBD/kg p.o), though this increase was still within reference range (2.4-5.0 g/dL) (Turner et al. 2023). This was the only significant finding, with all other serum chemistry parameters and physical examinations remaining unaffected (Turner et al. 2023). All other studies performed in horses report that CBD administration was well-tolerated, and no side effects were detected (Draeger et al. 2020; Interlandi et al. 2024; Ryan et al. 2021; Sánchez de Medina et al. 2023; St Blanc et al. 2022).

In human trials, CBD administration is generally well tolerated. Few studies report adverse events, mainly including decreased appetite, diarrhoea, sedation and somnolence (Chesney

et al. 2020). Sedation has been noted as a side effect, especially with high doses ranging from 10-20 mg CBD/kg administered once daily (Chesney et al. 2020).

In dogs, doses of up to 20 mg/kg BID p.o. are well-tolerated and associated with no to mild side effects (Di Salvo et al. 2023). Reported side effects include nausea, vomiting and loose stools, which could also be related to the formulation and taste of the respective CBD product. Less frequently, somnolence and lethargy were reported (Brioschi et al. 2020; Loewinger et al. 2022; Mogi and Fukuyama 2019). All side effects were self-limiting and appeared more frequently in doses > 10 mg CBD/kg (Di Salvo et al. 2023).

4 THESIS AIMS AND OUTLINE

This thesis aims to further investigate the pharmacokinetic properties of CBD and its effects on behaviour and stress responses in horses. The following hypotheses were proposed:

(1) Oral administration of a CBD-containing paste is well-tolerated in horses.

(2) Following oral administration, traces of CBD can be detected in blood and urine.

(3) Regular oral CBD administration has a modest stress-reducing effect in horses.

To address these hypotheses, a two-part study was designed. In the first part, a CBD-containing paste was applied as single oral administration in three escalating doses to horses. In the second part, the same CBD paste was administered every twelve hours over two weeks. A control group received a placebo paste in both study parts. Blood and urine samples were collected and analysed for traces of CBD and possible metabolites. Additionally, behavioural observations including evaluation of stress parameters were performed and compared between groups. The results are intended to provide further insights into the detection times of CBD in blood and urine, as well as its impact on equine behaviour. This information aims to contribute to a better understanding of the potential significance of CBD in equestrian sports.

5 PUBLICATIONS

5.1 Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses

Fabienne Eichler, Błażej Poźniak, Marc Machnik, Ina Schenk, Anke Wingender, Natalie Baudisch, Mario Thevis, Wolfgang Bäumer, Christoph Lischer, Anna Ehrle

Bibliographic Source

Eichler F, Poźniak B, Machnik M, Schenk I, Wingender A, Baudisch N, Thevis M, Bäumer W, Lischer C and Ehrle A (2023): Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses. Front Vet Sci: 10:1234551. https://doi.org/10.3389/fvets.2023.1234551

Contributions of first author

FE was majorly involved in the study design and planning of the project and was responsible for study execution including animal handling and data collection. FE performed the pharmacokinetic analyses and wrote the main draft of the manuscript.

Contributions of co-authors

All co-authors contributed to study design, planning, validation and writing of the manuscript. BP and WB were involved in the pharmacokinetic analyses and NLME model building. AW performed the drug assays under supervision of MM, IS and MT. NB supported the study execution. WB and CL were involved in formal analysis, methodology, project administration, supervision and validation. AE contributed to data curation, study execution, formal analysis, methodology, project administration, supervision and validation.

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 09 August 2023
DOI 10.3389/fvets.2023.1234551

OPEN ACCESS

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RECEIVED 04 June 2023
ACCEPTED 26 July 2023
PUBLISHED 09 August 2023

CITATION
Eichler F, Poźniak B, Machnik M, Schenk I, Wingender A, Baudisch N, Thevis M, Bäumer W, Lischer C and Ehrle A (2023) Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses.
Front. Vet. Sci. 10:1234551.
doi: 10.3389/fvets.2023.1234551

Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses

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Cannabidiol (CBD) products gain increasing popularity amongst animal owners and veterinarians as an alternative remedy for treatment of stress, inflammation or pain in horses. Whilst the use of cannabinoids is banned in equine sports, there is limited information available concerning CBD detection times in blood or urine. The aim of this study was to determine the pharmacokinetic properties of CBD following oral administration in the horse to assist doping control laboratories with interpreting CBD analytical results. Part 1: dose escalation study: Single oral administration of three escalating doses of CBD paste (0.2mg/kg, n=3 horses; 1mg/kg, n=3; 3mg/kg, n=5) with >7days wash-out periods in between. Part 2: multiple dose study: oral administration of CBD paste (3mg/kg, n=6) twice daily for 15days. Multiple blood and urine samples were collected daily throughout both studies. Following study part 2, blood and urine samples were collected for 2weeks to observe the elimination phase. Concentrations of CBD, its metabolites and further cannabinoids were evaluated using gas-chromatography/tandem-mass-spectrometry. Pharmacokinetic parameters were assessed via two approaches: population pharmacokinetic analysis using a nonlinear mixed-effects model and non-compartmental analysis. AUC_{0-12h} and C_{max} were tested for dose proportionality. During the elimination phase, the CBD steady-state urine to serum concentration ratio (Rss) was calculated. Oral CBD medication was well-tolerated in horses. Based on population pharmacokinetics, a three-compartment model with zero-order absorption most accurately described the pharmacokinetic properties of CBD. High volumes of distribution into peripheral compartments and high concentrations of 7-carboxy-CBD were observed in serum. Non-compartmental analysis identified a C_{max} of 12.17±2.08ng/mL after single administration of CBD (dose: 3mg/kg). AUC_{0-12h} showed dose proportionality, increase for C_{max} leveled off at higher doses. Following multiple doses, the CBD terminal half-life was 161.29±43.65h in serum. Rss was 4.45±1.04. CBD is extensively metabolized and shows high volumes of tissue distribution with a resulting extended elimination phase. Further investigation of the potential calming and anti-inflammatory effects of CBD are required to determine cut-off values for medication control using the calculated Rss.

KEYWORDS

CBD, cannabinoids, doping, drug control, equine, Monolix, PK, NLME model

1. Introduction

Medical cannabis and its extracted cannabinoids are used for the treatment of chronic pain, spasticity, epilepsy and anxiety in humans, and have been gaining popularity for similar indications in veterinary medicine in recent years (1–5). The cannabinoids most commonly known are cannabidiol (CBD), cannabidiolic acid (CBDA) and Δ9-tetrahydrocannabinol (THC) (6). CBD interacts with the CB_1- and CB_2 receptors of the endogenous endocannabinoid system and is described to have anti-inflammatory, relaxing, anti-convulsant and anxiolytic effects, whilst THC is the main agent responsible for the psychotropic characteristics of cannabis (7–14).

Pharmacokinetic studies in healthy dogs and cats, as well as clinical studies investigating the treatment of osteoarthritis, canine epilepsy and canine atopic dermatitis have confirmed positive outcomes with little side effects following the oral administration of CBD oil or paste (5, 15–23). Initial scientific reports of CBD application in horses described the treatment of mechanical allodynia, second intention wound healing and treatment for stereotypic behavior such as crib-biting (24–27). Subsequent studies started to analyze the pharmacokinetic properties of cannabinoids in horses and some studies reported positive therapeutic effects particularly for the treatment of chronic degenerative pain in horses (28–35).

Due to their potential analgesic and psychotropic properties, natural and synthetic cannabinoids are on the list of banned substances in most national and international equine sports associations including the FEI (Fédération Equestre Internationale) (36, 37). CBD and CBDA were moved to the FEI's list of controlled medications as specified substances in 2022 (36). The lipophilic properties of CBD and other cannabinoids can lead to the accumulation in organs and adipose tissue (5, 10, 38). The detection of synthetic cannabinoids in the context of doping control in horses has been described. There are, however, no further reports for detection times of CBD (36, 37, 39).

The aim of this study was to investigate the pharmacokinetic properties of CBD in horses following oral administration of a CBD containing paste, and to use the results for the interpretation of analytical findings following medication control in equestrian sports. The authors hypothesized that cannabinoids would have long retention times in equine biological matrices.

2. Materials and methods

2.1. Animals

Six Haflinger × Warmblood cross horses, including three mares and three stallions were included in the study. Mares and stallions were stabled in separate barns where the mares were kept in paddock boxes. All horses had *ad libitum* access to water, were fed hay and mineral feed and were led to pasture for 8 hours a day. The study was reviewed and approved by the competent authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347-12-2021).

2.2. CBD product

A paste containing 55% CBD (2,750 mg) and <0.2% THC (TAMACAN XL 55%®, 5,000 mg, Herosan healthcare GmbH, Austria) was used for oral medication. Further ingredients included naturally occurring phytocannabinoids, medium-chain triglyceride coconut oil, terpenes, flavonoids and beeswax. CBD and THC contents were analyzed and confirmed by an independent and internationally accredited anti-doping laboratory (Institute of Biochemistry, German Sport University Cologne, Cologne, Germany).

2.3. Dose escalation study

Initially, the CBD paste was administered in single escalating doses during three individual trials (trial 1: 0.2 mg/kg BWT, $n = 3$ horses; trial 2: 1 mg/kg, $n = 3$; trial 3: 3 mg/kg, $n = 6$). For better acceptance, the paste was inserted into a treat. There was a minimum washout period of 7 days in between trials. Prior to each trial, a physical examination was performed and a jugular vein catheter was aseptically placed. Blood samples were collected at the time points 0, 0.5, 1, 2, 4 and 12 hours (h) post medication for analysis of cannabinoid concentrations and for complete blood count (CBC; Diatron Abacus Junior 30 hematology analyser). Spontaneous urine samples were additionally collected at 2 and 12 h to be analyzed for cannabinoids. A repeated physical examination was performed between the time points 2–4 h following medication and horses were closely monitored for any signs of adverse reaction.

2.4. Multiple dose study

After a 25-day washout period, horses ($n = 6$) were administered oral CBD paste (3 mg/kg) every 12 hours for 15 days. Physical examinations were performed daily. Blood samples were obtained every day following oral medication at 2 and 11.5 h. CBC was performed daily at 2 h post administration (p.a.), and both the 2 and 11.5 h samples were analyzed for cannabinoid content. One spontaneous urine sample for cannabinoid analysis was collected from each horse between the time points 8–11.5 h. Serum kidney and liver biomarkers [blood urea nitrogen (BUN), creatinine (CREA), gamma-glutamyltransferase (GGT), glutamic oxaloacetic transaminase (GOT)] were assessed once a week (Fujifilm DRI-CHEM NX500i dry-chemistry analyser).

Following the final CBD oral application in the morning of day 15, blood samples were obtained at the time points 0, 0.5, 1, 2, 4 and 12 h and urine samples close to scheduled time points at 2 and 12 h for accurate monitoring of the drug elimination phase. Over the following 4 days (days 16–19), blood and urine samples were taken every 24 h

and subsequently every 36–48 h until day 33. CBC and serum kidney and liver biomarkers were assessed 1 week after trial end.

2.5. Cannabinoid analysis

Serum and urine samples were frozen and stored at −20°C until further processing. Quantitative analysis for cannabinoid concentrations was performed at an independent and internationally accredited anti-doping laboratory (Institute of Biochemistry, German Sport University Cologne, Cologne, Germany). All samples were analyzed by gas chromatography/tandem mass spectrometry (GC/MS/MS) for the presence of CBD, CBDA, cannabidivarin (CBDV), cannabigerol (CBG), THC, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (COOH-THC) and 11-hydroxy-Δ9-tetrahydrocannabinol (OH-THC). 7-carboxy-cannabidiol (COOH-CBD) and 7-hydroxy-cannabidiol (OH-CBD) were additionally assessed in serum and urine, respectively. Additional information on the sample preparation/extraction and instrumental conditions that were used in this study are summarized in the Supplementary material.

For the validation of analytical methods, parameters including precision, accuracy, selectivity, robustness, linearity, the lower limit of detection (LLOD), lower limit of quantification (LLOQ) and stability were determined. For selectivity, product ion scans were compared with spectra from the literature (40) or from spectra libraries. Three diagnostic product ions of each analyte were included in the acquisition method. Ten blank samples of each specimen (serum and urine) were prepared as described above and tested for interfering peaks at the expected retention time of the analytes. The samples showed no significant signals that could be attributed to the analytes. It was therefore concluded that the selectivity criteria of the employed method were met.

To evaluate the robustness of the method, 10 different samples of each specimen were spiked with 5 ng/mL of each cannabinoid, prepared and analyzed on two consecutive days. Potential effects of the different sample matrices (e.g., biological background interferences, specific gravity and pH differences, different horse characteristics like gender, race and age, potential haemolysis and analytical system performance) on the detectability (reproducibility of ion ratios, peak shape, signal intensity, signal-to-noise ratio and retention times) of each cannabinoid were controlled and documented. All samples showed signals for each analyte with reproducible signal intensities and ion ratios. Relative retention time shifts were within acceptable ranges <0.8% for all tested cannabinoids.

Linearity for all tested cannabinoids was examined by a series of spiked samples at 10 different concentrations in serum and urine over a concentration range considering the expected concentrations in p.a. samples. Area ratios of analyte and internal standard (y) were plotted against the analyte concentration (x) and a calibration curve ($y = ax + b$) was generated by linear least square regression with a weighting factor of $1/x$ or $1/x^2$ (Thermo Scientific Excalibur software version 4.0). The spiked concentration (theoretical concentration) was compared to the calculated concentration (measured concentration) of each calibrator. Correlation factors (R^2) were >0.98 for all calibration curves and measured concentrations were within the acceptance range of 85%–115% of the theoretical concentration for all cannabinoids.

A signal-to-noise ratio of ≥3 for the most abundant ion transition (quantifier ion) was used to determine the LLOD and a signal-to-noise ratio of ≥9 for the LLOQ in urine and serum. The LLOQ was verified by a six-fold determination of the estimated level to obtain the respective precision. The requirement for acceptance of the LLOQ was a coefficient of variation (CV) below 20%. Precisions were determined using 18 quality control (QC) samples which were spiked at low, medium and high concentrations quantified within 1 day ($n = 6$) and on three separate occasions ($n = 6 + 6 + 6$). The CV was established by 6 (intra-day precision) and 18 samples (inter-day precision). Respective concentrations of the QC samples and precisions for the four relevant cannabinoids in this study (CBD, CBDA, 7-COOH-CBD and 7-OH-CBD) are listed in Table 1. For the validation of the accuracy, QC samples ($n = 6$) each spiked at low, medium and high concentrations were quantified with a calibration curve. The means of measured values were compared with the theoretical values. Accuracies are expressed as relative errors (RE).

The stability was assessed by means of 12 serum and urine samples, each fortified with the tested cannabinoids at 5 ng/mL. One set of samples (6 serum and 6 urine) were prepared and analyzed on day 1, whereas the other spiked sample sets (6 serum and 6 urine) were stored at −20°C for 100 days and then quantified using freshly prepared calibrators. Stability was expressed as percentage ratio of the mean concentration at day 100 and the mean concentration at day 1.

Table 1 summarizes the resulting LLODs, LLOQs, precisions, accuracies and stabilities that were validated for each matrix and each compound.

TABLE 1 Validation results of the relevant cannabinoids in the present study.

Canna binoid	Matrix	LLOD (ng/mL)	LLOQ (ng/mL)	Intra-day precision CV (%) at 0.5/5.0/50 ng/mL	Inter-day precision CV (%) at 0.5/5.0/50 ng/mL	Accuracy RE (%) at 0.5/5.0/50 ng/mL	Stability [%]
CBD	Serum	0.1	0.2	9.4/3.9/1.6	6.9/3.7/4.1	9.9/1.6/6.5	63
	Urine	0.1	0.2	4.4/5.1/5.4	5.7/4.4/5.0	−0.4/−2.5/−6.6	83
CBDA	Serum	0.1	0.5	22.4/13.6/26.1	25.5/16.7/19.7	−2.8/−15.0/−12.4	51
	Urine	0.1	0.5	19.9/9.3/9.9	20.3/15.5/16.0	−19.4/−12.6/−7.1	45
7-COOH-CBD	Serum	0.1	0.2	12.5/5.8/6.7	12.5/6.1/4.2	1.4/2.7/−2.5	45
7-OH-CBD	Urine	0.1	0.2	10.0/4.9/3.9	9.4/11.4/6.6	2.5/−6.2/−3.7	79

LLOD, lower limit of detection; LLOQ, lower limit of quantification; CV, coefficient of variation; RE, relative error; CBD, cannabidiol; CBDA, cannabidiolic acid; 7-COOH-CBD, 7-carboxy-cannabidiol; 7-OH-CBD, 7-hydroxy-cannabidiol.

2.6. Pharmacokinetic analysis

2.6.1. Non-compartmental analysis

Non-compartmental analysis (NCA) was performed on serum CBD and its metabolites using PKanalix™ 2021R2 (MonolixSuite™ 2021R2, Lixoft, Antony, France). For the dose escalation study, the area under the curve from the first to the last sampling time point (AUC_{0-12h}), and value and time of maximum serum concentration (C_{max} and t_{max}) were calculated for CBD, 7-OH-CBD and 7-COOH-CBD and summarized as means and standard deviations (SD). The ratio of the AUC_{0-12h} for 7-OH-CBD/CBD and 7-COOH-CBD/CBD was additionally calculated. For the multiple dose study, the terminal half-life was determined for CBD and 7-COOH-CBD based on the last six time points.

2.6.2. Population pharmacokinetic analysis via a nonlinear mixed-effects model

To evaluate further pharmacokinetic parameters, serum CBD data was used to build a nonlinear mixed-effects model (NLME) applying the stochastic approximation expectation maximization (SAEM) algorithm with Monolix™ 2021R2. All CBD values from the dose escalation and the multiple dose studies were combined and fed into the software. The mean of the full posterior distribution was used to determine individual pharmacokinetic parameters. A mathematical model was written based on previous descriptions (41) with further refinements for veterinary purposes (42, 43):

$$y_{ij} = F\left(\varphi_i, t_{ij}\right) + G\left(\varphi_i, t_{ij}, \beta\right) \times \varepsilon_{ij}$$

$$\varepsilon_{ij} \sim N\left(0, \sigma^2\right), \varphi_i = h\left(\mu, \eta_i, \beta_i\right)$$

$$\varphi_i = \mu \times e^{\eta_i}, \eta_i \sim N\left(0, \Omega, \omega^2\right)$$

$$i = 1, \ldots, N, j = 1, \ldots, n_i$$

i stands for each single individual with N being the sum of all individuals. Sample times from 1 to n_i are described by j. y_{ij} is the CBD concentration observed per individual at time t_{ij}. The function $F(\varphi_i, t_{ij})$ predicts the individual concentration through parameter vector φ_i at timepoint t_{ij}. The associated residual error model G (φ_i, t_{ij}, β) contains the covariate β and is multiplied by the independent random variable ε_{ij}, which has a standard normal distribution including mean 0 and variance σ^2. The parameter vector φ_i was modelled as a function (h) of the mean population parameter μ with random variable η_i describing the individual variability and individual covariate β_i. A normal distribution of η_i with mean value 0, variance-covariance matrix Ω and variance ω^2 is assumed, leading to a log-normal distribution of individual parameters φ_i.

The final model was described by three compartments and zero-order absorption. The data set included oral administration only; therefore, the assessment of clearance (Cl) and volumes of distribution (V) was biased by the unknown bioavailability (F). Model parameters include the duration of the zero-order absorption (Tk0), systemic clearance (Cl/F), volume of distribution of a central (V1/F) and two peripheral (V2/F, V3/F) compartments, and intercompartmental clearances (Q2, Q3). Predicted C_{max} and t_{max} values were obtained from the tables generated for the individual predicted curves.

C_{max} were used to calculate the accumulation ratio (AR):

$$AR = \frac{C_{max\_multipledose}}{C_{max\_singledose}}$$

2.6.2.1. Parameter correlation estimates

To identify correlations between parameters which could aid model performance, scatterplots of η_i versus η_i-values for pharmacokinetic parameter estimates' pairs and the Pearson's correlation coefficient were evaluated. A t-test was performed to test statistical significance, defined as a p-value of <0.05. The obtained samples from the posterior distribution at the last SAEM iteration and the empirical Bayes estimates (EBEs) were assessed for parameter correlation, with the EBEs considered less relevant (43, 44). Correlations which fitted the defined selection criteria (see section 2.6.2.2 Model evaluation) were added to the final model.

2.6.2.2. Model evaluation

Numerical and graphical outputs (standard goodness-of-fit criteria, GOF) were used to evaluate the quality of the model (43, 44). To assess the SAEM algorithm, the stability of the parameter search and precision of the parameter estimates were examined for convergence through the relative standard error of the estimate (determined in the Fisher information matrix). Overparameterization was checked through the condition number of the eigenvalues. For graphical information, assessments were performed on individual observations vs. predictions, individual weighted residuals (IWRES), normalized predicted distribution errors (NPDE), visual predictive check (VPC) and individual fits. Distribution of the individual parameters and standardized random effects were examined through histograms and quantile-quantile plots. The random effects were evaluated for normal distribution using the Shapiro–Wilk test and the full posterior distribution of random effects and residuals. Models which performed satisfactorily were further inspected for precision of their respective parameter estimates and corrected Bayesian information criterion (BICc), before settling on a final model.

2.6.2.3. Addition of covariates

The horses' bodyweight was considered as a continuous covariate. The impact on model performance was assessed through the Pearson's correlation coefficient, Wald test and analysis of variance (threshold: p-value <0.05).

2.6.3. Dose proportionality

Pharmacokinetic parameters AUC_{0-12h} and C_{max} for CBD were tested for dose proportionality using the individual values

obtained from NCA and population pharmacokinetic analysis during the dose escalation study. Individual values were pooled for each parameter and fitted into a previously described power model (45, 46). Pharmacokinetic parameters (y) were log-transformed to apply a linear regression approach with dose as a covariate:

$$\log(y) = \mu + \beta \times \log(\text{dose})$$

The closer the β value is to 1, the more proportionally doses are aligned.

Additionally, the individual pharmacokinetic parameters were log-transformed and dose-normalized to test for significant differences (defined as p-value <0.05) between each trial using an analysis of variance (ANOVA) with a post-hoc Tukey test (Statistica 13, TIBCO, Palo Alto, CA, United States).

2.7. Application to medication control

Medication control in equestrian sports is either performed in urine or blood samples. To draw conclusions about the levels in urine from an existing blood sample of a medication, Toutain and Lassourd recommend estimating the steady-state urine to serum concentration ratio (Rss) of a potential drug (47). The concentrations of CBD in urine (Css_{urine}) and serum (Css_{serum}) were used to calculate the Rss during the elimination phase of the multiple dose study (pseudo-equilibrium condition) (47, 48):

$$\text{Rss} = \frac{\text{Css}_{\text{urine}}}{\text{Css}_{\text{serum}}}$$

3. Results

3.1. Horses

The horses' ages ranged from 3 to 16 years (median = 11 years) and the body weight was 488 ± 55 kg. One horse developed a jugular vein thrombophlebitis during the third trial of the dose escalation study and was excluded, putting the final number of horses participating in trial three to $n = 5$. As the inflammation subsided over the following days, it was considered safe to include the horse in the subsequent multiple dose study. Oral application of the CBD product was well tolerated. Physical examinations showed no irregularities and mean assessments of CBCs, kidney and liver biomarkers remained within reference range throughout both trials in all horses (Table 2). Maximum white blood cell (WBC) count was 13.15 10^9/L (reference range (RR): 5–10 10^9/L). Values for BUN below RR were between 6.9–9.3 mg/dL (RR: 9.4–23.5 mg/dL) and for CREA between 0.8–0.9 mg/dL (RR: 0.9–1.5 mg/dL). GGT remained within RR in all samples. GOT was 387 IU/L in one horse (RR: 165–358 IU/L) after 7 days of treatment (Table 2).

TABLE 2 Mean ± standard deviation of WBC count, kidney and liver biomarkers during multiple administrations of CBD paste (3 mg/kg po) twice daily over two weeks with subsequent sample collection.

Parameter (RR)	Baseline	Day 7	Day 14	Day 21
WBC (5–10 10^9/L)	9.0 ± 2.2	7.8 ± 1.6	7.9 ± 2.0	7.6 ± 1.9
Number of horses out of RR	$n = 2/6$	$n = 1/6$	$n = 0/6$	$n = 1/6$
BUN (9.4–23.5 mg/dL)	10.1 ± 1.1	11.0 ± 0.9	10.0 ± 1.0	11.3 ± 2.2
Number of horses out of RR	$n = 2/6$	$n = 0/6$	$n = 1/6$	$n = 2/6$
CREA (0.9–1.5 mg/dL)	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
Number of horses out of RR	$n = 0/6$	$n = 1/6$	$n = 1/6$	$n = 1/6$
GGT (10–50 IU/L)	22.3 ± 2.9	23.5 ± 4.8	23.0 ± 2.4	20.5 ± 3.3
Number of horses out of RR	$n = 0/6$	$n = 0/6$	$n = 0/6$	$n = 0/6$
GOT (165–358 IU/L)	290.2 ± 38.6	298.0 ± 47.5	288.8 ± 29.7	295.7 ± 21.8
Number of horses out of RR	$n = 0/6$	$n = 1/6$	$n = 0/6$	$n = 0/6$

The number of horses in each group with serum levels outside of RR are also reported. RR, reference range; WBC, white blood cell; BUN, blood urea nitrogen; CREA, creatinine; GGT, gamma-glutamyltransferase; GOT, glutamic oxaloacetic transaminase.

3.2. Pharmacokinetic analysis

3.2.1. Non-compartmental analysis

3.2.1.1. Dose escalation study

Concentration curves with mean ± standard deviations of CBD and its main metabolites 7-COOH-CBD and 7-OH-CBD in serum and urine are shown in Figure 1. In the first trial (dose: 0.2 mg/kg), CBD and 7-COOH-CBD were found in serum and CBD and 7-OH-CBD were found in urine. In the second trial (dose: 1 mg/kg), CBD, 7-OH-CBD and 7-COOH-CBD were identified in serum, but 7-OH-CBD remained below the LLOQ. CBD, 7-OH-CBD, CBDA, CBDV and CBG were detected in urine with CBDA levels being below the LLOQ (Supplementary Figure S1). In the third trial (dose: 3 mg/kg), CBD, 7-OH-CBD and 7-COOH-CBD were identified in serum. In urine, CBD, 7-OH-CBD, CBDA, CBDV and CBG were detected (Figure 1; Supplementary Figure S1). CBDA levels were again below LLOQ. Table 3 presents the parameters $AUC_{0-12\,h}$, C_{max} and t_{max} assessed in the NCA and the $AUC_{0-12\,h}$ ratio between CBD and its metabolites 7-OH-CBD and 7-COOH-CBD. C_{max} and t_{max} could not be determined for 7-COOH-CBD, as the concentration curves have not decreased sufficiently by time point 12 h (Figure 1).

3.2.1.2. Multiple dose study

CBD, 7-OH-CBD, 7-COOH-CBD, CBDV, THC and OH-THC were identified in serum. 7-OH-CBD concentrations were below the LLOQ from 60 h after last CBD administration onwards (Figure 2). CBDV and THC were detected in concentrations around the LLOQ throughout the trial [C_{max}(CBDV) = 0.39 ng/mL; C_{max}(THC) = 0.70 ng/mL]. CBDV and THC values were below the LLOQ at 4 h and 12 h

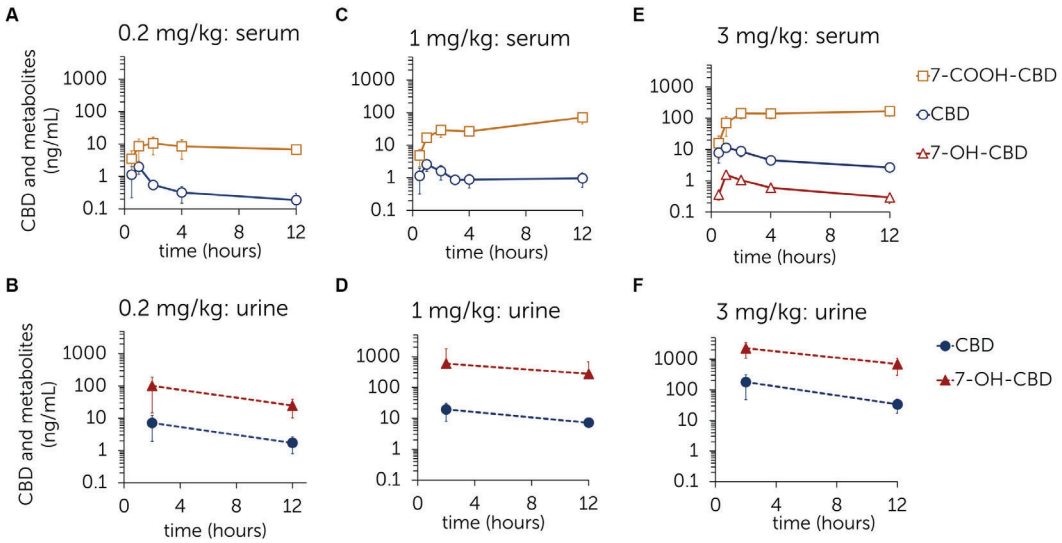

FIGURE 1
Mean ± standard deviation of serum and urine concentrations of cannabidiol (CBD) and the metabolites 7-hydroxy-cannabidiol (7-OH-CBD) and 7-carboxy-cannabidiol (7-COOH-CBD) after single oral administration of CBD paste in three different doses [0.2 mg/kg **(A,B)**; 1 mg/kg **(C,D)**; 3 mg/kg **(E,F)**].

TABLE 3 Mean ± standard deviation of pharmacokinetic parameters for CBD and metabolites following single oral administrations of CBD paste during dose escalation study, derived from NCA.

Parameter	First trial (0.2 mg/kg, *n* = 3)	Second trial (1 mg/kg, *n* = 3)	Third trial (3 mg/kg, *n* = 5)
CBD			
AUC_{0-12h} (h·ng/mL)	4.45 ± 2.52	15.46 ± 6.08	59.53 ± 13.54
C_{max} (ng/mL)	1.98 ± 0.99	2.58 ± 1.25	12.17 ± 2.08
t_{max} (hr)	1 ± 0	1 ± 0	1.1 ± 0.55
7-COOH-CBD			
AUC_{0-12h} (h·ng/mL)	106.95 ± 65.68	571.02 ± 194.33	1768.38 ± 450.86
Ratio: $\frac{AUC_{0-12h}(7-COOH-CBD)}{AUC_{0-12h}(CBD)}$	21.09 ± 3.19 (2109.15%)	38.78 ± 7.82 (3877.88%)	31.02 ± 6.38 (3102.13%)
7-OH-CBD			
AUC_{0-12h} (h·ng/mL)	—	—	6.62 ± 1.86
Ratio: $\frac{AUC_{0-12h}(7-OH-CBD)}{AUC_{0-12h}(CBD)}$	—	—	0.10 ± 0.03 (10.23%)
C_{max} (ng/mL)	—	—	1.42 ± 0.37
t_{max} (hr)	—	—	1.4 ± 0.55

NCA, non-compartmental analysis; CBD, cannabidiol; 7-COOH-CBD, 7-carboxy-cannabidiol; 7-OH-CBD, 7-hydroxy-cannabidiol; AUC_{0-12h}, area under the serum concentration-time curve (from time point 0 to 12 h); C_{max}, maximum concentration; t_{max}, time of maximum concentration.

after last CBD administration. OH-THC concentrations remained mostly below the LLOQ except for the time points 202.5 h (0.26 ng/mL) and 314 h (0.27 ng/mL) (Supplementary Figure S2).

In urine, CBD, 7-OH-CBD, CBDA, CBDV and CBG were identified. CBDA concentrations fell below the LLOQ 36.5 h after the last CBD administration. CBG and CBDV values remained below the LLOQ 131 h and 248 h after the last CBD administration, respectively (Figure 2; Supplementary Figure S2).

The terminal half-life for CBD and 7-COOH-CBD in serum was calculated based on the last six time points (132–360 h) after the last CBD administration. For CBD, the terminal half-life was 161.29 ± 43.65 h and for 7-COOH-CBD, it was 79.85 ± 18.03 h.

3.2.2. Population pharmacokinetic analysis

A three-compartment model best described the pharmacokinetic properties of CBD in horses. Residual error was described through a

combined 1 error model, containing a constant and proportional term. Numerical and graphical outputs were evaluated for GOF and predictive power. Diagnostic plots are shown in Figures 3–6. The visual predictive check (VPC) shows close prediction of median values (Figure 4). Empirical data for the 10th and 90th percentile are deviating from their respective confidence intervals (CI) at around

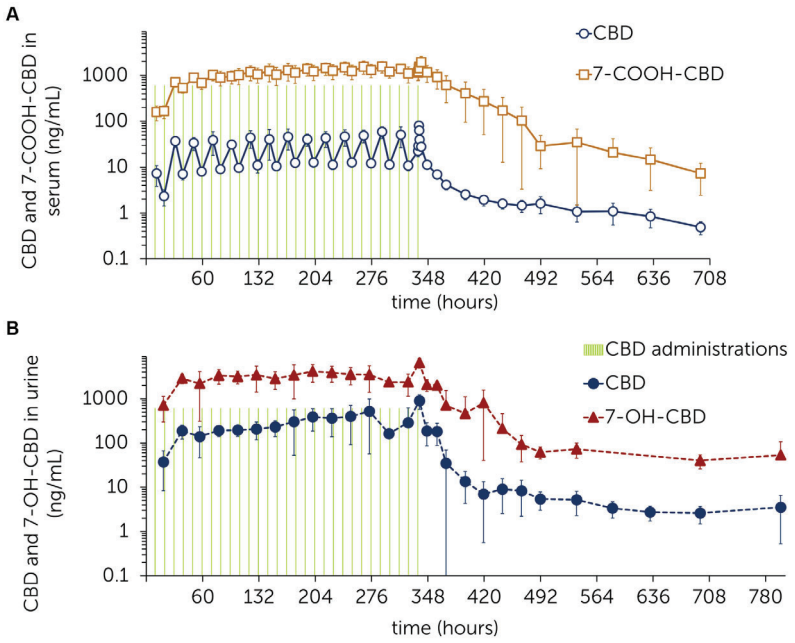

FIGURE 2
Mean ± standard deviation of serum **(A)** and urine **(B)** concentrations of cannabidiol (CBD) and the metabolites 7-hydroxy-cannabidiol (7-OH-CBD) and 7-carboxy-cannabidiol (7-COOH-CBD) following multiple administrations of CBD paste (3 mg/kg po) twice daily over 2 weeks with subsequent sample collection.

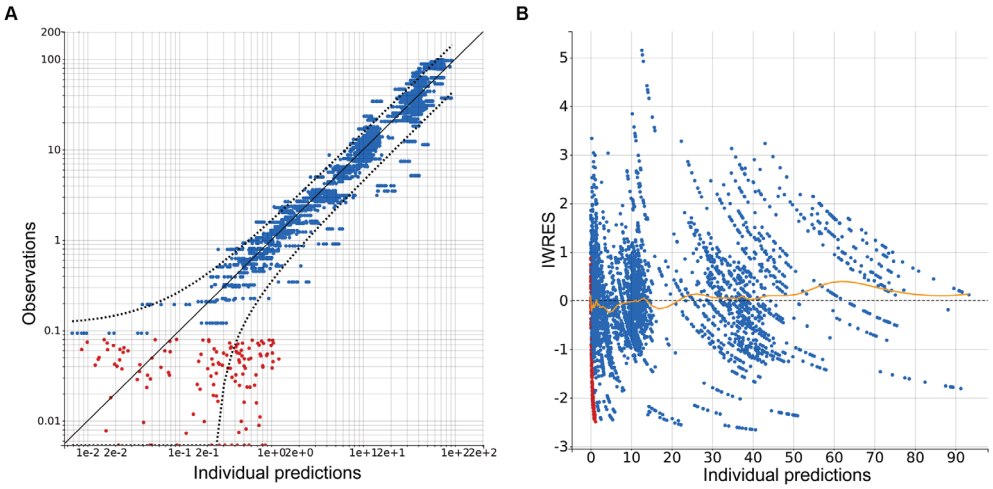

FIGURE 3
Diagnostic plots extracted from the three-compartment model following population pharmacokinetic analysis. **(A)** Plot of observations vs. individual predictions. Blue dots indicate observations, red dots indicate censored data, black line—identity line; dotted black line represents the 90% prediction interval. Outliers proportion was 10.54%. **(B)** Scatterplot of individual weighted residuals (IWRES) vs. individual predictions. Blue dots indicate observations, red dots indicate censored data, spline is marked with a yellow line.

220 h and 350 h, respectively. Exemplary graphs depicting individual predictions are presented in Figure 5.

Inter-occasion variability (IOV) was not included as it was similar to the individual variability and, due to the relatively small number of subjects, led to a low precision of estimates. Profiles were therefore treated as separate individuals. Random effects were estimated for Cl/F, V1/F, Q3 and V3/F. For the other parameters, the population value was used as the random effects were converging to zero and were insufficiently assessed in all individuals. Correlating V3/F and Q3 further improved the fit of the model (Figure 6).

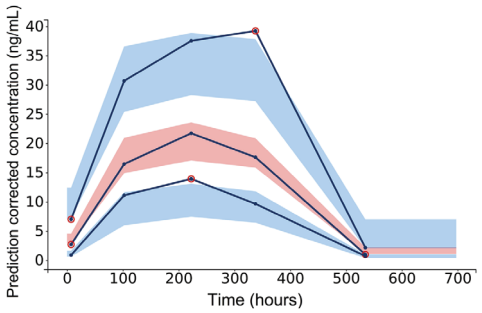

FIGURE 4
Diagnostic plot extracted from the three-compartment model following population pharmacokinetic analysis: visual predictive check for CBD concentrations in serum. Empirical data [10th, 50th (median) and 90th percentile] are marked by solid lines. Outlier dots are circled in red. Shaded areas mark the 90% confidence intervals for corrected prediction of the median (red) and the 10th and 90th percentile (blue).

Table 4 presents the final pharmacokinetic parameters derived through the population pharmacokinetic approach. The low relative standard error (RSE) values confirm accurate assessment for the population parameter estimates. The low eigenvalue ratio (29.07, derived from the Fisher information matrix) and low shrinkage (< 20%, see Table 4) indicate that the model was not over-parameterized. The values for volume of distribution in the central (V1/F) and peripheral compartments (V2/F and V3/F) suggest a very high distribution of CBD as well as retention in tissues. The estimation of convergence accounts for the model's robustness.

Bodyweight as an added covariate did not show any effect on the pharmacokinetic parameters and was excluded from the final model.

AUC_{0-12h} as an additional output and C_{max} and t_{max} (extracted from individual fits) are presented in Table 5. Values are shown in relation to the parameters derived from the NCA (Table 3).

To calculate the accumulation ratio (AR), C_{max} from each day of the multiple dose study was summarized to a mean of 38.39 ± 8.89 ng/mL. Mean C_{max} from trial 3 of the dose escalation study was 14.61 ± 5.08 ng/mL. AR was therefore 2.63.

3.2.3. Dose proportionality

The power model equation revealed the β value for the NCA parameter AUC_{0-12h} to be 0.99 and for C_{max} to be 0.72. For the population pharmacokinetic parameters, the β value for AUC_{0-12h} was 0.93 and 0.80

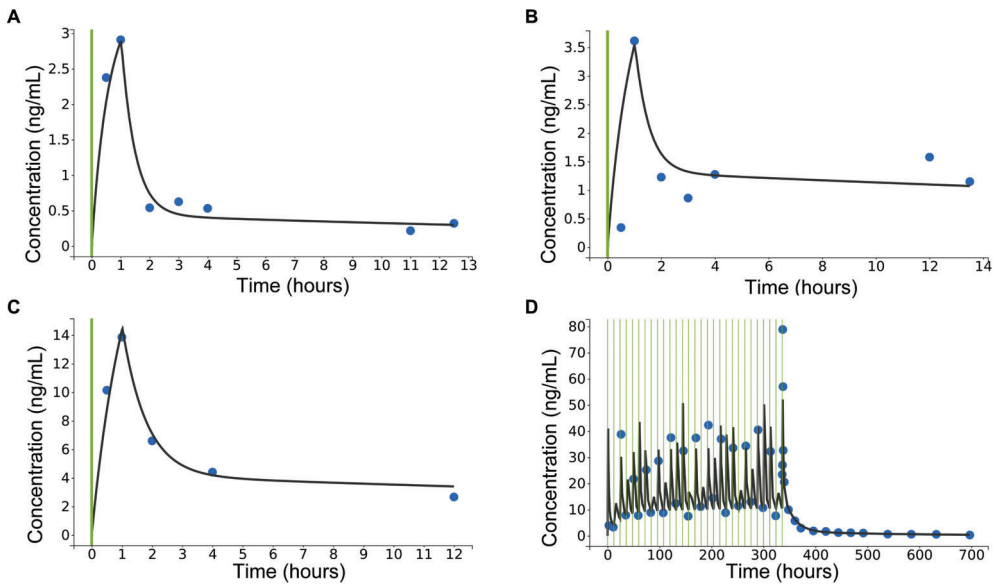

FIGURE 5
Diagnostic plots extracted from the three-compartment model following population pharmacokinetic analysis: exemplary individual predictions for concentrations of cannabidiol (CBD) in serum after single oral administration of CBD paste in three different doses [**(A)**: 0.2 mg/kg po; **(B)**: 1 mg/kg po; **(C)**: 3 mg/kg po], and **(D)**: following multiple administrations of CBD paste (3 mg/kg po) twice daily over 2 weeks with subsequent sample collection. Green lines represent CBD administrations, blue dots are observed data points and black lines are individual fits.

for C_{max}. As the individual values were pooled for this approach, the inter-individual variability through a CI was not determined.

An ANOVA with a post-hoc Tukey test identified a significant difference between the dose-normalized C_{max} obtained from NCA between trial 1 (0.2 mg/kg) and trial 2 (1 mg/kg) ($p = 0.014$). Trials 2 and 3 (3 mg/kg), and trials 1 and 3 showed no statistically significant differences ($p = 0.334$, $p = 0.123$). Similarly, there were no statistically significant differences between the other pharmacokinetic parameters.

3.3. Application to medication control

Between 60 to 360 h after the last CBD administration in the multiple dose study, a pseudo-equilibrium condition was reached (Figure 7) (47, 48). The steady-state urine to serum concentration ratio (Rss) was calculated from the mean concentration values: Rss = 4.45 ± 1.04.

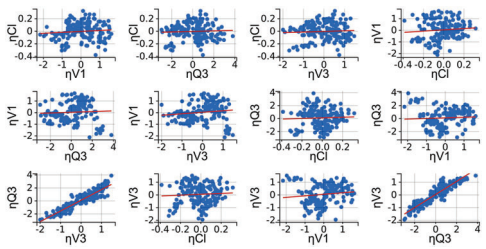

FIGURE 6
Diagnostic plot extracted from the three-compartment model following population pharmacokinetic analysis: Correlation plots of the random effects (η_i). Correlation was applied when correlation coefficients were estimated to be high and met the threshold for inclusion (Pearson's correlation test, $p < 0.05$). Linear regressions are presented as red lines.

4. Discussion

Investigation of the pharmacokinetic properties of CBD following repeated oral administration identified a rapid increase of the CBD serum concentration with an extended elimination phase of CBD and its metabolites. These findings indicate an extensive metabolism of CBD with prolonged tissue retention.

The oral administration of CBD paste was well-tolerated by all horses in the current study and side effects such as gastrointestinal intolerance were not observed. A previous study reported mildly elevated liver enzymes after multiple oral administrations of a CBD-infused oil (1 mg/kg and 3 mg/kg) in horses (30). Another study reported decreased creatinine levels and higher gamma-glutamyltransferase levels, although still within normal reference range (49). In this study, only occasional, slight shifts out of RR without associated clinical signs were observed in WBC count, kidney and liver biomarkers.

Like in other equine and small animal investigations, the pharmacokinetic analysis showed a rapid increase of CBD in serum following oral administration (15, 16, 28, 29, 50–55). The values for C_{max} were similar to those calculated in other studies (28–31, 33). In contrast, the AUC_{0-12h} values obtained here differ significantly. This is caused by the fact that in the previous studies AUC were determined over longer time periods (up to 264 h) (28–31, 33). The AUC_{0-12h} values reported for the single dose part of the current study are much lower as the time dimension of this parameter is terminated at 12 h. It was not possible to credibly determine relative bioavailability for the used formulation. This would require calculating $AUC_{0-\infty}$ and compare it with the results of previously published studies. As for the single dose administration, the terminal portion of the curve was not sufficiently captured to assess $AUC_{0-\infty}$.

A long elimination phase for CBD was shown during the multiple dose study (Figure 2). Based on the visual inspection of the individual log-linear concentration-time profiles, the terminal phase of elimination started approx. 132 h after the last CBD administration. Therefore, only the following data-points were used for the calculation of the elimination half-life. As previous studies have

TABLE 4 Population pharmacokinetic parameters of orally administered CBD paste in four different equine trials.

	Population value	SE	RSE (%)	Omega	SE	RSE (%)	Shrinkage (%)
Population parameter estimates (unit)							
Tk0 (h)	1.02	0.11	10.5	—	—	—	—
Cl/F (L/h/kg)	10.75	0.7	6.53	0.15	0.049	33.6	15.1
V1/F (L/kg)	77.13	20.11	26.1	0.83	0.18	22.1	2.27
Q2 (L/h/kg)	1.35	0.14	10.2	—	—	—	—
V2/F (L/kg)	313.17	50.63	16.2	—	—	—	—
Q3 (L/h/kg)	38.23	15.72	41.1	1.48	0.47	31.8	9.11
V3/F (L/kg)	241.98	67.77	28.0	0.85	0.24	28.0	12.9
Residual error							
a	0.07	0.021	29.8	—	—	—	—
b	0.33	0.016	5.04	—	—	—	—

Data derived from three separate trials with single doses of 0.2 mg/kg (administered to $n = 3$ horses), 1 mg/kg ($n = 3$) and 3 mg/kg ($n = 5$) and a multiple dose study with a dose of 3 mg/kg administered twice daily over 15 days ($n = 6$). CBD, cannabidiol; SE, standard error; RSE, relative standard error, Tk0, duration of the zero-order absorption; Cl/F, total body clearance; V1/F, volume of distribution in the central compartment; V2/F, volume of distribution in the first peripheral compartment; V3/F, volume of distribution in the second peripheral compartment; Q2, clearance between V1 and V2; Q3, clearance between V1 and V3; F, bioavailability.

TABLE 5 Mean ± standard deviation of pharmacokinetic parameters for CBD and metabolites following single oral administrations of CBD paste during the dose escalation study, derived from the individual fits of the population pharmacokinetic model.

	First trial (0.2 mg/kg, n = 3)	Second trial (1 mg/kg, n = 3)	Third trial (3 mg/kg, n = 5)
AUC_{0-12h} (h·ng/mL)	4.99 ± 1.56	13.64 ± 5.33	58.56 ± 12.98
C_{max} (ng/mL)	1.82 ± 0.83	3.10 ± 1.27	14.61 ± 5.08
t_{max} (hr)	1.01 ± 0.01	1.02 ± 0.03	1.02 ± 0.01
Ratio: $\frac{\text{Parameter}(CBD_{Pop\_PK})}{\text{Parameter}(CBD_{NCA})}$			
$\frac{AUC_{0-12h}(CBD_{Pop\_PK})}{AUC_{0-12h}(CBD_{NCA})}$	1.20 ± 0.24	0.86 ± 0.11	0.98 ± 0.09
$\frac{C_{max}(CBD_{Pop\_PK})}{C_{max}(CBD_{NCA})}$	0.92 ± 0.08	1.21 ± 0.21	1.18 ± 0.29
$\frac{t_{max}(CBD_{Pop\_PK})}{t_{max}(CBD_{NCA})}$	1.01 ± 0.01	1.02 ± 0.03	1.12 ± 0.52

Values are presented as ratios to the parameters derived from the non-compartmental analysis (Table 3). CBD, cannabidiol; AUC_{0-12h}, area under the serum concentration-time curve from time point 0 to 12 h; C_{max}, maximum concentration, t_{max}, time of maximum concentration; NCA parameters, parameters derived from non-compartmental analysis; CBD_{Pop_PK}, parameter for CBD derived through population pharmacokinetics; CBD_{NCA}, parameter for CBD derived through non-compartmental analysis.

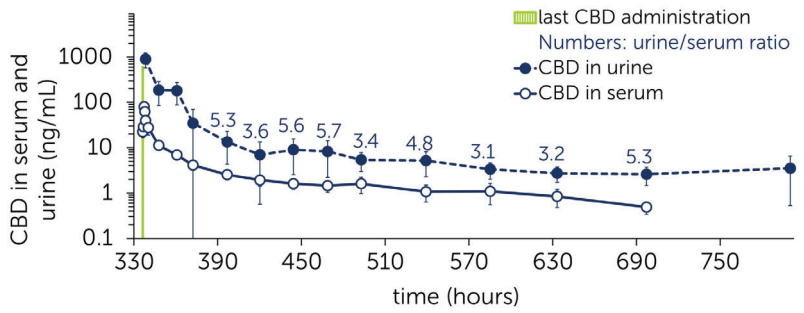

FIGURE 7
Mean ± standard deviation of serum and urine concentrations of cannabidiol (CBD) during the elimination phase. Last CBD administration (dose: 3 mg/kg) to six horses at time point 336 h following multiple administrations of CBD paste (3 mg/kg po) twice daily over 2 weeks. Numbers present the urine/serum ratio between respective time points.

derived the terminal half-life from earlier time points, values are difficult to compare (28–31, 33). The very long elimination phase of CBD suggests a high volume of distribution into different tissue compartments.

Previous studies hypothesized, that CBD is subject to a high first pass effect with a considerable pre-systemic metabolism in the liver (29, 33, 56). The extensive metabolism of CBD into 7-COOH-CBD is mirrored by the high ratio of their AUC_{0-12h} (Table 3). In comparison, the AUC_{0-12h} ratio between CBD and 7-OH-CBD is substantially lower. To the best of the authors knowledge, research detailing the exact steps of CBD metabolism in horses is currently not available. In humans, 7-OH-CBD is further metabolized to 7-COOH-CBD (57, 58). Based on this information, the low serum value of 7-OH-CBD in the current study may be explained by the partial metabolism into 7-COOH-CBD. In line with other reports, higher concentrations of 7-OH-CBD were detected in urine (29). Further research investigating the exact metabolic pathway of CBD in horses following oral administration would be of great interest.

For data derived from the NCA and the population pharmacokinetic approach, CBD ratios for AUC_{0-12h}, C_{max} and t_{max} were close to 1, confirming that the individual fits calculated in the NLME model are close to the actual concentrations measured (Table 5).

Values for volumes of distribution and clearance [both over bioavailability (F)] were derived through the population pharmacokinetic analysis. Although the study design did not

include intravenous administration to precisely estimate the true clearance and volumes of distribution, the application of NLME modelling allowed the pooling of data into a single robust model, despite different study designs (single vs. multiple administrations) and dose levels. Volumes of distribution over F were high in the central and the two peripheral compartments (Table 4). Other studies in horses and dogs describe similar values based on non-compartmental analysis, even though doses and study protocols differ slightly (28, 51). Values are especially high for V2/F and V3/F in the current study, suggesting a very high distribution and tissue retention of CBD. This observation is further supported by the low inter-compartmental clearance value Q2 (1.35 L/h/kg) between V1 and V2. One reason might be the lipophilic properties of CBD, as confirmed by several canine and human studies (5, 10, 38). The high volumes of distribution could however be misleading, as the population pharmacokinetic model does not account for the extensive metabolism of CDB to 7-COOH-CBD. The authors chose to exclude the additional metabolite data out of the NLME modelling, as its inclusion and the subsequent classification of CBD as a parent drug did not produce a satisfying and stable model. The relatively small sample size and the lack of data for intravenous administration necessitated the choice of a simpler but much more stable model that met all the goodness-of-fit criteria.

The estimated clearance value of 10.75 L/h/kg is comparable to one study (33), but lower than the results from other equine studies that were also obtained using oral data with an unknown F (29, 30). Comparing clearance values with those from other species proved to be difficult, as very few reports exist and values are declared in L/h instead of L/h/kg (51, 56). One study reports a very high variance for clearance of CBD and its metabolites in dogs (59).

Considering all species, only few reports compare oral and intravenous administrations of CBD to calculate *F*. *F* has been described to be 7.92% and 14% in horses, putting it in a similar range with findings in humans (6%) and dogs (13%–22.28%) (31, 33, 51, 56, 60). The low *F* values further confirm the high first-pass-effect of CBD with extensive pre-systemic metabolism and a high liver extraction ratio, as described in humans (72%) (29, 56).

The visual predictive check of the population pharmacokinetic analysis shows good agreement with the median values, but there is a noticeable deviation of the 10th and the 90th percentile's empirical data from the 10% and 90% CI at approximately 220 and 350 h after the first CBD administration (Figure 4). These deviations are likely caused by the differing concentration values of CBD in serum in one horse. This particular horse showed consistently higher values than the median. This may have been caused by interindividual variability or over-dosing of the CBD paste due to variation of the horse's bodyweight. The authors decided not to exclude this horse from the dataset, as the other values were not affected by the described deviation. Moreover, such high variability in the internal exposure is not uncommon for drugs with low bioavailability, therefore the authors believe that this dataset may reflect the real-life situation well.

As the CBD product used in this study was extracted from the cannabis plant (*Cannabis sativa*), further phytocannabinoids were identified during the serum and urine analysis. Values for CBDV and THC in serum were very low throughout the study and reached levels just above LLOQ. In urine, CBDV and CBG were detected in higher concentrations. There is very little information available on the potential effects of these phytocannabinoids. One study reports CBDV to have an anti-convulsant effect in mice and rats (61). CBG's influence on pain perception has been tested in mouse models (62, 63) and its pharmacokinetic properties have recently been described in dogs (64). Another study showed that CBG decreases the intraocular pressure in cats (65). The potential therapeutic use of CBG for the treatment of human diseases like multiple sclerosis has additionally been suggested (66).

During the multiple dose study, the steady state for CBD was reached at day 2 (Figure 2). The accumulation ratio (AR) under steady state for CBD in serum was 2.63. In humans, an AR of 2–5 is considered to indicate moderate drug accumulation (67). The time it takes to eliminate CBD from the bloodstream is therefore moderately long compared to the dosing interval (12 h). This observation might be helpful in establishing dosing patterns or time points for maximum efficacy. Concentration values in urine are less stable but are also showing fair consistency from day 2 onwards. As urine samples were collected as spot samples, values must be evaluated with caution.

The dose proportionality evaluated with an ANOVA did not identify any statistically significant differences in the dose-normalized parameters between trials, except for C_{max} obtained from the NCA between trial 1 (dose: 0.2 mg/kg) and 2 (dose: 1 mg/kg). Since C_{max} between trial 1 and trial 3 (dose: 3 mg/kg), and trials 2 and 3 did not differ significantly, this variability might be explained in part by the low bioavailability and small sample size in the dose escalation study. In the power model, C_{max} from the NCA had the lowest β value (0.72), confirming the variability and therefore possible lack of proportionality as seen in the ANOVA. β values for $AUC_{0-12\,h}$ were very close to 1, suggesting that CBD administered as a paste within the studied dose range leads to a dose proportional exposure with the extent of absorption remaining unchanged. On the other hand, the rate of absorption appears to decrease with higher doses as the increase for C_{max} becomes less linear (exemplified by the comparatively small β values). This observation may further support the choice of zero-order absorption as a model parameter in the population pharmacokinetic analysis. However, the small number of individuals within the specific dose groups and the high variability in exposure reduce the statistical significance of these results.

Graphical illustration shows that CBD concentrations in serum and urine achieve a pseudo-equilibrium condition during the elimination phase (Figure 7) (48). The values exemplify that CBD concentrations detected in serum can be translated to residual concentrations in urine by the calculated Rss. Whether these residual concentrations influence a horse's performance and must be subject to medication control, remains unclear. Specific cut-off values for a drug can be defined through a nonexperimental approach, where irrelevant drug plasma concentrations (IPC) and irrelevant drug urine concentrations (IUC) are calculated (47). IPC and IUC are based on the average effective plasma concentration (EPC), which is derived from the standard dose (per dosing interval) and bioavailability. As no standard dose with a proven effect for CBD in horses has been defined so far, EPC, IPC and IUC were not calculated in the current study.

Limitations of the study include the lacking assessment of the inter-occasion variability (IOV) due to the small sample size and testing of only one CBD product through only one route of administration. Further studies may evaluate varying CBD doses administered intravenously to obtain precise estimates for clearance, volumes of distribution and bioavailability, and to gain a better understanding of CBD's metabolism.

5. Conclusion

This study confirms the extensive metabolism of CBD and suggests a prolonged retainment in tissues resulting in the extended elimination phase of CBD and its metabolites. The oral administration of CBD paste proved to be well-tolerated and did not cause any side effects at a maximum dose of 3 mg/kg following oral administrations twice daily over 2 weeks. A population pharmacokinetic model pooling data from both single and multiple dose studies has been successfully developed. Whilst the steady-state urine to serum concentration ratio (Rss) was defined, future research analyzing the effect of CBD on behavioral parameters and anti-inflammatory responses are required. Once an effective therapeutic dose is established, specific cut-off values for medication control may be established further. Until then, the administration of CBD products to sport horses should be treated with caution.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the competent authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347-12-2021).

Author contributions

FE and AE were involved in all parts of the project. CL, WB, MM, and IS contributed to study design, planning of the project, and data analysis. FE and NB were responsible for study execution including animal handling and data collection. AW performed the drug assays under supervision of MT, MM, and IS. BP and FE performed the pharmacokinetic analyses. FE wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The study was funded by the Freie Universität Berlin, the German Equestrian Federation (FN), and Herosan healthcare GmbH. Herosan healthcare GmbH was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. We acknowledge support by the Open Access Publication Fund of the Freie Universität Berlin.

Acknowledgments

The authors would like to thank Franziska Berger, Julia Bolk, Lena Haas, Katharina Jessat, Anja Kühnel, Jenny Ries, Clara Schubert, and Elisa Zimmermann for their support with horse handling and laboratory work, and the whole team at the Equine Center Bad Saarow, especially Johannes Handler, for their support during the practical part of the study. The authors further acknowledge the support of Conny Pint and Daniela Wimmer, and sincerely thank Philine Bank for initiating the project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1234551/full#supplementary-material

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5.2 Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2)

Fabienne Eichler, Anna Ehrle, K. Charlotte Jensen, Natalie Baudisch, Hannah Petersen, Wolfgang Bäumer, Christoph Lischer, Mechthild Wiegard

Bibliographic Source

Eichler F, Ehrle A, Jensen KC, Baudisch N, Petersen H, Bäumer W, Lischer C and Wiegard M (2023): Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2). Front Vet Sci: 10:1305868. https://doi.org/10.3389/fvets.2023.1305868

Contributions of first author

FE was majorly involved in the study design and planning of the project and was responsible for study execution including animal handling and data collection. FE performed the formal analysis including statistics of behavioural observations, heart rate and hear rate variability parameters and wrote the main draft of the manuscript.

Contributions of co-authors

All co-authors contributed to study design, planning, validation and writing of the manuscript. AE was involved in data curation, study execution, formal analysis, methodology, project administration, supervision and validation. KCJ supported the statistical analysis. NB contributed to study execution. HP performed data analysis of behavioural parameters under supervision of MW. WB, CL and MW contributed to formal analysis, methodology, project administration, supervision and validation.

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 11 December 2023
DOI 10.3389/fvets.2023.1305868

OPEN ACCESS

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RECEIVED 02 October 2023
ACCEPTED 06 November 2023
PUBLISHED 11 December 2023

CITATION
Eichler F, Ehrle A, Jensen KC, Baudisch N, Petersen H, Bäumer W, Lischer C and Wiegard M (2023) Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2). *Front. Vet. Sci.* 10:1305868. doi: 10.3389/fvets.2023.1305868

Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2)

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Cannabidiol (CBD) products have been proposed to exert stress- and anxiety-relieving effects in animals. Despite the increasing popularity of CBD for veterinary use, the available research detailing the effects of CBD in horses is limited. The aim of this study (part 1 of 2) was to analyze stress parameters via behavioral observations and heart rate monitoring in healthy horses following single oral administration of a CBD containing paste in different doses. Study products were two pastes for oral administration, one containing CBD and one containing no active ingredient. Pastes were applied as single administrations in consecutive trials with escalating dosages (doses: 0.2, 1.0, 3.0 mg CBD/kg) to a treatment (trial 1: $n = 3$, trial 2: $n = 3$, trial 3: $n = 5$ horses) and a control group (trial 1: $n = 3$, trial 2: $n = 3$, trial 3: $n = 6$ horses) with minimum wash-out periods of seven days in between. Behavioral parameters were evaluated using video recordings to score the levels of sedation including the horses' reactions to acoustic and visual stimuli. Facial expression was assessed using photographs. Evaluation was based on the previously described facial sedation scale for horses (FaceSed) and the Horse Grimace Scale. For baseline values, identical observations were recorded on the day before each paste administration. Both paste administration and behavioral evaluation were performed double blinded. Cardiac beat-to-beat (R-R) intervals were continuously recorded throughout the trial and assessed using heart rate and heart rate variability parameters. Statistical analysis included comparison between treatment and control group over escalating doses and time points using linear mixed models. The CBD paste was well tolerated, and no side effects were observed. Analysis of sedation scores and facial expressions did not indicate significant differences between treatment and control group over the escalating doses. The heart rate was neither reduced, nor were significant changes in heart rate variability observed compared to the control group. Main limitation of this study is the small sample size. Further research is required to determine adequate doses and indications for the use of CBD products in horses.

KEYWORDS

behavior, CBD, equine, FaceSed, Horse Grimace Scale, sedation score

1 Introduction

Cannabidiol (CBD) belongs to the most well-known compounds of *Cannabis* plants and is gaining increasing attention in the field of veterinary medicine. Unlike Δ^9-tetrahydrocannabinol (THC), CBD does not exhibit psychoactive properties (1, 2) but has been tested for analgesic, anti-inflammatory and anti-convulsant effects in companion animals (3–8). Additionally, the impact of CBD on anxiety and stress relief is currently under investigation. In humans, stress and anxiety are the most common indications for CBD use (9).

Mechanisms of action include various pathways: CBD may act as a ligand on serotonin$_{1A}$ (5-HT$_{1A}$) receptors (10–14) and inhibits the deactivation of endogenous cannabinoids such as anandamide (AEA) (15–17). AEA is a ligand of the endocannabinoid (eCB) system which regulates emotional responses and can reduce anxiety (12, 18, 19). CBD may also influence cannabinoid type 1 (CB$_1$) receptors of the eCB system as an indirect agonist by increasing membrane fluidity and therefore modulating the constitutional activity of CB$_1$ (12, 20, 21).

In humans and rodents, CBD has been reported to decrease heart rate and to show anxiolytic effects (9, 22–25). However, results remain inconsistent, as other studies could not confirm these findings to the same extent (26–29). Further effects of CBD include sedation, which has been reported in humans (30, 31). In dogs, surveys among US veterinarians and pet owners have reported that sedation is a perceived side effect following CBD or hemp supplementation (32–34). It was additionally suggested that CBD supplementation may decrease stress-related aggressive behavior (1). Another study could not identify significant alteration in daily activity or quality of sleep in dogs (35). There are few reports detailing the effect of CBD on equine behavior: One study found a reduction of reactivity without any significant effect on the heart rate (36). Other reports showed no effect of CBD on ataxia, sedation scores or overall equine behavior (37, 38). Two case reports described CBD as an effective treatment for stereotypic behavior such as crib-biting and mechanical allodynia (39, 40). The effect of CBD on horses is of particular interest as all cannabinoids are on the list of prohibited substances issued by the international governing body of equestrian sports (FEI, Fédération Equestre Internationale) due to their assumed psychotropic properties (41).

The aim of this study was to analyze stress levels via behavioral observations and heart rate monitoring in healthy horses following oral administration of a CBD containing paste to further validate equine behavior under the influence of CBD medication. The authors hypothesized that increasing CBD doses would have a moderately calming effect in horses.

2 Materials and methods

2.1 Animals

Twelve Haflinger × Warmblood cross horses, including seven mares and five stallions, were randomly assigned to a treatment or a control group ($n = 6 + 6$). Horses' age varied between 3 to 16 years (median: 11 years) in the treatment group and 10 to 26 years (median: 10.5 years) in the control group. Mares and stallions were housed separately with mares having free paddock access. All horses were fed hay and mineral feed, and spent 8 h a day on pasture. The study was approved by the competent authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347–12–2021).

2.2 Study products

Study products were two pastes (treatment and control). The treatment paste contained 55% full spectrum CBD plant extract, medium-chain triglyceride (MCT) coconut oil, naturally occurring phytocannabinoids, terpenes, flavonoids and beeswax (TAMACAN XL 55%®, Herosan healthcare GmbH, Austria). The THC content was below 0.2%. The control paste contained MCT oil and beeswax only. The ingredients of both pastes were analyzed, and concentrations of the contents were confirmed by an independent and internationally accredited anti-doping laboratory (Institute of Biochemistry, German Sport University Cologne, Germany). Pastes were labeled "A" or "B" by the manufacturer before shipment to conceal their formulations. People handling the horses, i.e., caretakers and sample takers, were unaware of the horses' group assignment.

2.3 Dose escalation study

The study was divided into three trials with administration of CBD paste in escalating doses (trial 1: 0.2 mg CBD/kg; trial 2: 1 mg CBD/kg; trial 3: 3 mg CBD/kg). Doses were selected based on the manufacturer's recommendation and the current literature (36, 38). The first two trials were performed with three horses in each group ($n = 3$ treatment + 3 control) and close attention was paid to the occurrence of possible side effects. The third trial (3 mg CBD/kg) was subsequently performed with all twelve horses ($n = 6$ treatment + 6 control). The day before each trial, horses were physically examined and a jugular vein catheter was aseptically placed. On the day of trial, the paste (A or B) was orally administered at 6:30 am. For better acceptance, the paste was inserted into a treat. To determine pharmacokinetic parameters of CBD administration in horses, multiple blood and urine samples were taken throughout the trials from all horses (42).

Equine behavior was recorded for the subsequent evaluation of a sedation score by an independent observer at time points 0, 1, 2, 4 and 12 hours (h) after paste administration (Figure 1). The occurrence and the depth of sedation was determined based on the observed position of the horse's head and the reaction to acoustic and visual stimuli (Table 1). Acoustic stimuli included a clicker as it is used for positive reinforcement training as well as the crackling noise of a plastic bag. As a visual stimulus, a pink cloth was attached to a stick and waved in front of the horse's face. Reactions to the stimuli were video recorded. Additionally, photographs were taken for subsequent assessment of the facial expressions. Expressions were rated based on the horse's orbital openings, position of ears, visibility of chewing muscles, position of lips and dilation of nostrils (Table 2).

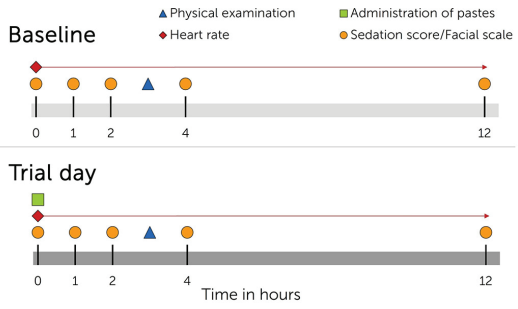

FIGURE 1
Timeline showing interventions for each cannabidiol (CBD) oral medication trial. **Upper panel**, day before trial start. **Lower panel**, trial day. Trials were repeated three times with single administration of escalating CBD doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW) and wash-out periods of minimum seven days in between trials.

Each horse's heart rate (HR) was continuously recorded throughout the trials using a Polar® H10 heart rate sensor (Polar® Electro Oy, Kempele, Finland). The sensor was attached to an electrode belt which spanned around the horse's chest. To enhance skin contact and signal transmission, the coat was trimmed and moisturized with water over the heart base between the 4th and 5th intercostal space where the electrodes were positioned. Each sensor was connected to a mobile device via Bluetooth to document the cardiac beat-to-beat (R-R) intervals with the Polar® Equine App (Version 1.2.1, Polar® Electro, Kempele, Finland).

Repeated physical examination was performed 2–4 h following paste administration, and blood samples were obtained for white blood cell (WBC) count.

Baseline values including recordings of equine behavior and heart rate were obtained in the same pattern as described on the day before each trial for comparative analysis (Figure 1). Trials were divided by wash-out periods of at least seven days.

2.4 Assessment of behavioral observations

Evaluation of the video recordings was based on a previously described sedation score (43). For assessment of the photographs, a facial expression scale was developed based on the facial sedation scale for horses (FaceSed) (44) and the Horse Grimace Scale (45). The described parameters were modified according to the reactions and expressions observed in the study animals (Tables 1, 2). Videos and photographs of each horse were randomly arranged and blinded assessment was performed by one person who was experienced in equine behavior studies but not actively involved in any of the trials. For each horse, stimulus and time point, the five parameters of the sedation score were summed up, resulting in scores ranging from 5 to 20 (Table 1). The scores of the three stimuli were then summed up to a total for each horse and time point, resulting in a total sedation score ranging from 15 to 60. For the facial expression scale, parameters were similarly added up to a possible total sum of 6–18 for each time point and each individual horse. A score of 10 was given when the eyes were open, the ears forward pointing, the chewing muscles moderately present, the lips loosely touching and the nostrils non-dilated (Table 2). High scores represent a deeper relaxation or sedation.

2.5 Assessment of heart rate and heart rate variability

Heart rate (HR) and heart rate variability (HRV) were analyzed using the software Kubios® HRV Standard (ver. 3.5, Kubios® Oy, Kuopio, Finland). Parameters included the mean HR in beats per minute (bpm), the root mean square of successive beat-to-beat differences (RMSSD in milliseconds, ms) and the standard deviation of normal-to-normal beat-to-beat intervals (SDNN, ms). Automatic beat correction was applied to remove artifacts (threshold: very low, 0.3 s). Each recording period was divided into sections of 15 min as previously described (46).

2.6 Statistical analysis

Data were recorded in Microsoft Excel® (Version 2304) and statistical analysis was performed with SPSS® Statistics 27 (IBM®, NY, USA). First, data was analyzed descriptively: The value for each total sedation score and the sedation scores of the three stimuli were displayed in bar charts (mean + standard deviation). For the inductive analysis, the difference between the total sedation score at baseline and during the trial was calculated for each horse and time point (ranging from −45 to +45). Similarly, the differences between score on baseline and trial day were calculated for the facial expression scale (ranging from −12 to +12). The effects of the dose levels on the differences between baseline and trial day of the total sedation score were analyzed using linear mixed models. Individual horses were assigned as subjects, dose levels as fixed effects (reference = control group; trial 1 = 0.2 mg CBD/kg; trial 2 = 1 mg CBD/kg; trial 3 = 3 mg CBD/kg) and time points as random effects (0 h; 1 h; 2 h; 4 h; 12 h). Residuals were visually inspected for normal distribution. The level of significance was $p < 0.05$. For the facial expression scale, the differences between baseline and trial day were calculated and tested for an effect of dose levels using a linear mixed model as described above.

For HR, RMSSD and SDNN parameters, the first eight 15-minute sections (total of two hours) post paste administration were selected for analysis as CBD blood concentrations reached a maximum here (42). To test for an effect of dose levels on the parameters, linear mixed models were calculated as described above.

To identify systematic differences between baseline and trial day values of HR, RMSSD and SDNN within the treatment group over time, linear mixed models for each outcome were calculated with trials (reference = baseline; trial 1 = 0.2 mg CBD/kg; trial 2 = 1 mg CBD/kg; trial 3 = 3 mg CBD/kg) as fixed effects. The following analysis was performed as described above with individual horses

TABLE 1 Sedation score developed for behavioral observations following single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg; 1 mg CBD/kg; 3 mg CBD/kg), based on the sedation score by Poller et al. (43).

Head position	
1	Lower lip at height of shoulder joint or higher
2	Lower lip between shoulder and olecranon
3	Lower lip between olecranon and carpal joint
4	Lower lip at carpal joint or lower
Reaction to stimulus: head movement	
1	Focus directed toward stimulus, jerky aversion
2	Focus directed toward stimulus, aversion, then refocusing on stimulus
3	Focus directed toward stimulus, slight aversion
4	Indifference/no reaction
Reaction to stimulus: ear movement	
1	Ears pointed, obvious flickering of ears, steady response to stimulus
2	Moderate flickering of one or both ears
3	Slight flickering of one or both ears
4	Indifference/no reaction
Reaction to stimulus: Chewing	
1	Chewing movement is interrupted and does not continue
2	Chewing movement is repeatedly interrupted and recontinued
3	Chewing movement is interrupted once and recontinued
4	Indifference/no interruption of chewing
Reaction to stimulus: body movement	
1	Moving back more than one step, turning away
2	Moving back one step, head jerking
3	Jerking/lifting/averting of head
4	Indifference/no reaction
Total sum for EACH stimulus: 5 - 20	
Total sum for ALL stimuli: 15 - 60	

A total sum was calculated for each stimulus (clicker, bag, cloth) and for all stimuli.

TABLE 2 Facial expression scale developed for behavioral observations following single oral administration of cannabidiol (CBD) paste in three escalating doses (0.2mg CBD/kg; 1mg CBD/kg; 3mg CBD/kg), based on the FaceSed (44) and Horse Grimace Scale (45).

Orbital opening	
2	Eyes completely open
3	Eyes partially open (> 50%)
4	Eyes almost/completely closed (< 50%)
Position of ears	
1	Pinned back
2	Forward pointed, position of attention
3	Asymmetrical; one ear hanging
4	Wide opening between ear tips
Chewing muscles	
1	Strained/obviously present
2	Moderately present
3	Not present
Lips	
1	Strained mouth
2	Loose touching of lips
3	Slight relaxation of one lip
4	Pronounced relaxation/hanging of one lip
Nostrils	
1	Dilated, outer ring clearly visible
2	Non-dilated nostrils
3	Small nostrils, relaxed outer ring
Total sum: 6 - 18	

as subjects, dose levels as fixed effects and time points as random effects.

3 Results

3.1 Animals

The horses' body weight was on average 488 ± 55 kg in the treatment group and 443 ± 56 kg in the control group. During the first two trials, no side effects such as gastrointestinal intolerances were observed following paste application and it was considered safe to proceed with trial three. During trial three, one mare developed signs of a jugular vein thrombophlebitis and was excluded, resulting in five remaining horses in the treatment group to complete trial three ($n = 5 + 6$). Over all trials, the WBC count remained close to reference range with only mild WBC elevation (maximum WBC in the treatment group = 11.63 10^9/L) (Table 3).

TABLE 3 Mean ± standard deviation of white blood cell (WBC) count after single oral administration of a cannabidiol (CBD) containing paste in three trials.

Parameter (Ref)	First trial (0.2 mg CBD/kg)	Second trial (1 mg CBD/kg)	Third trial (3 mg CBD/kg)
Control group			
WBC count (5–10 10^9/L)	7.43 ± 0.98	6.88 ± 0.38	7.79 ± 1.28
Number of horses out of Ref (Value out of Ref)	$n = 0/3$	$n = 0/3$	$n = 1/6$ (10.31 10^9/L)
Treatment group			
WBC count (5–10 10^9/L)	10.49 ± 0.68	9.79 ± 1.33	7.97 ± 2.19
Number of horses out of Ref (Value out of Ref)	$n = 1/3$ (11.17 10^9/L)	$n = 1/3$ (11.63 10^9/L)	$n = 1/5$ (11.60 10^9/L)

The number of horses with serum levels outside of the reference range (Ref) are reported for each group.

3.2 Behavioral observations

3.2.1 Sedation score

For all three trials, graphical illustration of the statistical data using bar charts did not identify a clear trend for higher or lower sedation scores between groups or dose levels (Figure 2, Supplementary Figures S1–S3). During trial 1, overall scores for baseline values ranged from 29.3 ± 1.3 to 40.3 ± 3.9 at all time points in the treatment group. Overall scores for trial day values ranged from 29.5 ± 5.5 to 45.3 ± 2.5 at all time points. In the control group, values ranged between 27.8 ± 5.3 to 34.5 ± 6.3 at baseline and between 23.2 ± 1.0 to 39.9 ± 10.8 on trial day. No trend was observed for values being generally higher or lower at certain time points in either group.

During trial 2, baseline values ranged from 32.0 ± 6.7 to 41.8 ± 8.3 and trial day values from 38.8 ± 10.0 to 44.3 ± 9.9 in the treatment group. All values were higher on trial day than at baseline as exemplified by graphical illustration. In the control group, baseline values were between 28.4 ± 6.2 to 36.8 ± 7.3 and trial day values between 28.8 ± 10.4 to 37.7 ± 10.2. Values were higher on trial day than the corresponding baseline values at time points 2, 4 and 12.

During trial 3, baseline values in the treatment group were between 31.1 ± 5.5 to 37.9 ± 12.2 and trial day values between 29.8 ± 10.8 to 39.2 ± 11.4. In the control group, baseline values ranged from 28.0 ± 6.6 to 41.7 ± 9.9 and trial day values from 31.3 ± 6.7 to 35.4 ± 4.1. No trend was observed for values being generally higher or lower at certain time points in either group.

Linear mixed models with escalating doses as fixed effects did not identify significant differences between the total sum of sedation scores in the treatment and control group [$P(F) = 0.527$]. Even during trial 2, the difference was not significant [$P(F) = 0.180$]. Similarly, the individual scores were not significantly influenced by escalating doses for stimulation with a clicker [$P(F) = 0.196$], crackling of a plastic bag [$P(F) = 0.442$] or waving with the pink cloth [$P(F) = 0.915$]. Estimates for random effects for the total sum were: $\beta = 25.9$ [95% confidence intervals (CI) = 6.7, 100.6; standard error (SE) = 17.9], for clicker: $\beta = 7.7$ (95% CI = 2.9, 20.4; $SE = 3.8$) and for plastic bag: $\beta = 1.3$ (95% CI = 0.0, 126.8; $SE = 3.0$). Random effects were not estimated for visual stimulation with a cloth. For the total sum, 21.7% of variability was accounted to differences between time points. For stimulation with a clicker and plastic bag, time points as random effects were attributed to 32.6 and 4.7% of variability, respectively.

3.2.2 Facial expression scale

Examples for scoring of the facial expressions are shown in Supplementary Table S1. Graphical illustration of sedation scores is shown in Figure 3.

During trial 1, overall scores for baseline values ranged from 10.0 ± 0.0 to 12.0 ± 2.2 at all time points in the treatment group. Overall scores for trial day values ranged from 9.7 ± 0.5 to 10.3 ± 0.5 at all time points. All values were equal or lower on trial day than at baseline. In the control group, baseline values ranged from 8.5 ± 1.5 to 10.7 ± 0.9 and from 10.0 ± 0.0 to 12.7 ± 2.1 on trial day. All values were equal or higher on trial day than at baseline. In this trial, the most notable differences between baseline and trial day were found at time point 1 (treatment group: 12.0 ± 2.2 to 10.3 ± 0.5) and time point 12 (control group: 10.7 ± 0.9 to 12.7 ± 2.1).

During trial 2, baseline values in the treatment group were between 9.8 ± 0.6 to 10.7 ± 0.6 and trial day values between 10.0 ± 0.0 to 10.7 ± 0.5. In the control group, baseline values ranged from 10.0 ± 0.0 to 10.7 ± 0.9 and trial day values from 10.0 ± 0.0 to 10.5 ± 0.7. No trend was observed for values being generally higher or lower at certain time points in either group.

During trial 3, baseline values in the treatment group ranged from 10.0 ± 0.8 to 10.7 ± 0.7 and trial day values from 10.0 ± 0.0 to 10.4 ± 0.5. In the control group, baseline values ranged from 10.0 ± 0.0 to 10.2 ± 0.9 and trial day values from 10.0 ± 0.0 to 10.4 ± 0.8. No trend was observed for values being generally higher or lower at certain time points in either group.

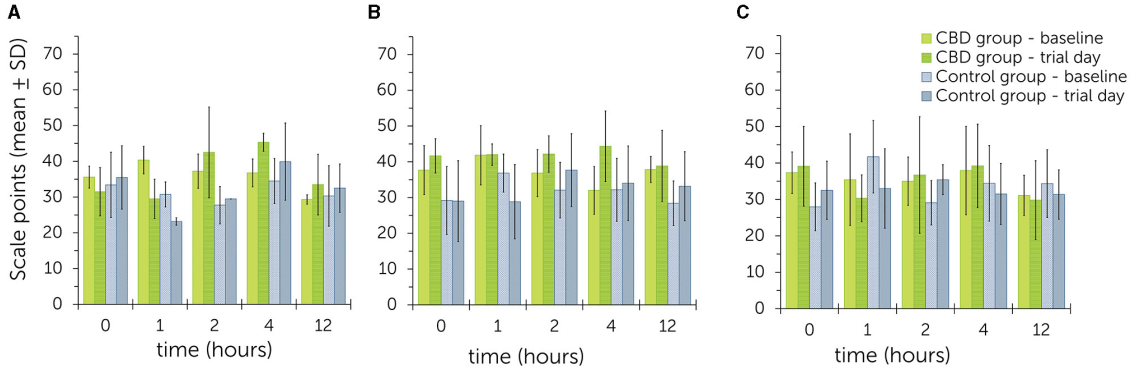

FIGURE 2
Summed up sedation scores after acoustic and visual stimulations (clicker, plastic bag, pink cloth) following single oral administration of cannabidiol (CBD) paste in escalating doses (**A**: 0.2 mg CBD/kg; **B**: 1 mg CBD/kg; **C**: 3 mg CBD/kg) - comparison between values obtained on baseline and trial day for the treatment and control group. Higher scale points relate to a higher level of sedation (Table 1). No significant differences were found between treatment and control group over all three trials.

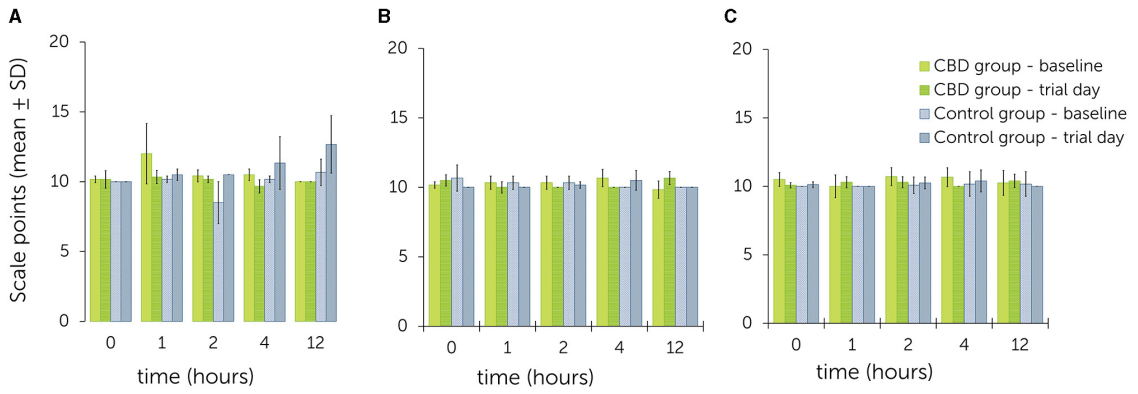

FIGURE 3
Facial expression scale following single oral administration of cannabidiol (CBD) paste in escalating doses (**A**: 0.2 mg CBD/kg; **B**: 1 mg CBD/kg; **C**: 3 mg CBD/kg) - comparison between values obtained on baseline and trial day for the treatment and control group. Higher scale points relate to a higher level of sedation (Table 2).

TABLE 4 **Fixed effects estimates for the comparison of differences (Δ) between score levels reached on a facial expression scale on baseline and trial days [single oral administration of cannabidiol (CBD) paste in three escalating doses (0.2 mg CBD/kg; 1 mg CBD/kg; 3 mg CBD/kg)].**

Parameter	Regression coefficient (β)	95% confidence intervals (CI)	Standard error (SE)	p-value
Δ Score levels (facial expression scale)				
Intercept	0.3	0.0, 0.7	0.2	0.077
Control group	Reference			
Trial 1 (0.2 mg CBD/kg)	−0.9	−1.6, −0.1	0.4	0.021
Trial 2 (1 mg CBD/kg)	−0.4	−1.1, 0.4	0.4	0.344
Trial 3 (3 mg CBD/kg)	−0.6	−1.2, 0.0	0.3	0.065

The linear mixed model did not identify a significant effect of escalating CBD doses on the facial expression scale when compared to the control group [$P(F) = 0.080$]. Considering the fixed effects estimates, a significant effect was evident between trial 1 and the control group ($p = 0.021$) (Table 4). The estimate for the random effects was $\beta = 0.1$ (95% CI = 0.0, 27.4; $SE = 0.2$) with 3.3% of variability attributed to differences between time points.

3.3 Heart rate and heart rate variability

3.3.1 Comparison between treatment and control group

Mean HR and HRV values are shown in Table 5. On trial days, the mean HR in the first 2 h post paste administration was between 42.1 ± 8.6 bpm to 45.4 ± 7.5 bpm in the treatment group, and between 41.3 ± 8.2 bpm to 44.4 ± 9.8 bpm in the control group.

RMSSD values ranged between 122.7 ± 48.8 ms and 152.9 ± 36.6 ms in the treatment group, and 137.1 ± 35.4 ms and 151.6 ± 29.3 ms in the control group. For SDNN, mean values were between 105.4 ± 22.8 ms and 163.1 ± 48.4 ms in the treatment group, and between 135.7 ± 64.4 ms and 156.8 ± 49.6 ms in the control group. Graphical representations of mean HR, RMSSD and SDNN are shown in Figures 4–6 (trial days) and Supplementary Figures S4–S6 (baseline).

Statistical analysis using linear mixed models found that doses as fixed effects had no significant impact on HR [$P(F) = 0.139$], RMSSD [$P(F) = 0.104$] and SDNN [$P(F) = 0.202$]. A significant difference could not be identified even between the highest CBD dose (3 mg CBD/kg) and the control group (HR: $p = 0.377$; RMSSD: $p = 0.189$; SDNN: $p = 0.734$) (Table 6).

For HR, the estimate for the random effects was $\beta = 31.5$ (95% CI = 15.1, 65.7; $SE = 11.8$). Differences between time sections are accounted for 44.1% of variability. The RMSSD estimate was $\beta = 607.0$ (95% CI = 262.0, 1406.3; $SE = 260.2$) and 33.2% of variability was attributed to time sections. For SDNN, β was 1107.0 (95% CI = 456.3, 2685.8; $SE = 500.6$). Time sections were associated with 33.7% of variability.

3.3.2 Comparison between baseline and trial day within the treatment group

Mean HR values showed no trend indicating a consistent increase or decrease from baseline to trial day in the treatment group (Table 5). Mean RMSSD and SDNN values showed a consistent increase from baseline to trial day during all trials, except

TABLE 5 Mean ± SD values for HR, RMSSD and SDNN values from the first 2 h after single oral cannabidiol (CBD) paste administration with corresponding baseline values. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

Parameter	Treatment group – baseline (mean ± SD)	Treatment group – trial day (mean ± SD)	Control group – baseline (mean ± SD)	Control group – trial day (mean ± SD)
HR (bpm)				
Trial 1 (0.2 mg CBD/kg)	30.2 ± 2.9	45.4 ± 7.5	no data	41.4 ± 4.6
Trial 2 (1 mg CBD/kg)	45.3 ± 7.0	43.3 ± 4.1	43.2 ± 7.2	41.3 ± 8.2
Trial 3 (3 mg CBD/kg)	42.6 ± 6.6	42.1 ± 8.6	39.0 ± 4.4	44.4 ± 9.8
RMSSD (ms)				
Trial 1 (0.2 mg CBD/kg)	127.7 ± 51.2	152.9 ± 36.6	no data	151.6 ± 29.3
Trial 2 (1 mg CBD/kg)	112.7 ± 33.8	123.6 ± 30.6	151.3 ± 39.4	137.1 ± 35.4
Trial 3 (3 mg CBD/kg)	113.8 ± 40.0	122.7 ± 48.8	151.0 ± 61.7	140.9 ± 48.2
SDNN (ms)				
Trial 1 (0.2 mg CBD/kg)	140.8 ± 44.6	163.1 ± 48.4	no data	156.8 ± 49.6
Trial 2 (1 mg CBD/kg)	110.1 ± 41.0	105.4 ± 22.8	154.4 ± 71.1	146.0 ± 49.7
Trial 3 (3 mg CBD/kg)	104.6 ± 44.7	131.0 ± 61.1	121.5 ± 38.5	135.7 ± 64.4

SD, standard deviation; HR, heart rate; RMSSD, root mean square of successive R-R interval differences; SDNN, standard deviation of normal-to-normal R-R intervals; bpm, beats per minute; ms, milliseconds.

for a decrease in SDNN values during trial 2 (110.1 ± 41.0 ms to 105.4 ± 22.8 ms).

Examination of the differences between baseline and trial day values identified no significant effect for HR [$P(F) = 0.136$] over all three trials but found significant effects for RMSSD [$P(F) = 0.016$] and SDNN [$P(F) < 0.001$]. Both significant findings can be attributed to trial 1 and trial 3 (Table 7). Estimates for random effects for HR were: $\beta = 13.1$ (95% CI = 5.0, 34.1; $SE = 6.4$), for RMSSD: $\beta = 768.5$ (95% CI = 399.6, 1478.2; $SE = 256.5$) and for SDNN: $\beta = 1052.6$ (95% CI = 537.88, 2060.1; $SE = 360.6$). For HR, RMSSD and SDNN values, differences between time sections are accounted for 22.5%, 40.6% and 39.6% of variability, respectively.

4 Discussion

Investigation of stress parameters in healthy horses, including behavioral observations and heart rate monitoring, following oral administration of a CBD containing paste in escalating doses did not identify consistently significant differences when compared to a control group.

CBD products are marketed for a variety of conditions in animals including improving general wellbeing and having a calming and stress-relieving effect (3–8). Sedation is a reported side effect associated with CBD application in humans and dogs (30–34, 47). To assess sedation in horses, multiple scoring systems have been proposed but are mainly aimed at testing sedatives such as detomidine or acepromazine (43, 48, 49). As levels of sedation in this study were not pronounced and scoring based on established scales did not produce satisfying results, a previously described sedation scale (43) was adjusted to the behavior exhibited by the horses in the current study (37). The dose levels tested in this study (0.2 mg CBD/kg, 1 mg CBD/kg, 3 mg CBD/kg) did not result in any significant difference in sedation scores after acoustic or visual stimulation compared to the control group. This is in agreement with a previous report where sedation levels were scored in horses following CBD administration (37). In this report, pellets containing 150 mg CBD (∼ 0.29 mg CBD/kg) were fed over 56 days with no significant difference in sedation levels detected when compared to a control group. In humans, sedation was described as a side effect after daily oral intake of a total of 600 mg CBD over 6 weeks (47). Future studies may investigate whether higher dose administrations lead to more significant signs of sedation in horses.

Photographs were taken to assess the potential influence of CBD on equine facial expression. Existing scoring systems including FaceSed and Horse Grimace Scale (HGS) were modified to suit the purpose of the current report, as CBD administration did not produce sedation levels comparative to those depicted in the FaceSed scale (44, 45). Horses additionally displayed facial expressions described in the HGS, like strained mouth and chewing muscles. As the horses included in the current study did not undergo any painful procedures, similar expressions were interpreted as signs of stress. Expressions related to annoyance, such as pinned-back ears, were also exhibited. Only the modified scores of trial 1 (0.2 mg CBD/kg) were significantly different when compared between treatment and control group ($p = 0.021$). Score levels were higher at baseline than on trial day in the treatment group at time points 1, 2 and 4, whereas score levels in the control group were consistently lower at baseline than on trial day (Figure 3). As this result is the only significant event in this study part and comparisons with higher dose administrations did not produce significant results, its relevance should be interpreted with caution.

CBD reduces anxiety and stress by acting as a direct or indirect agonist on 5-HT_{1A}- and CB_1-receptors (10–14, 20). Stress

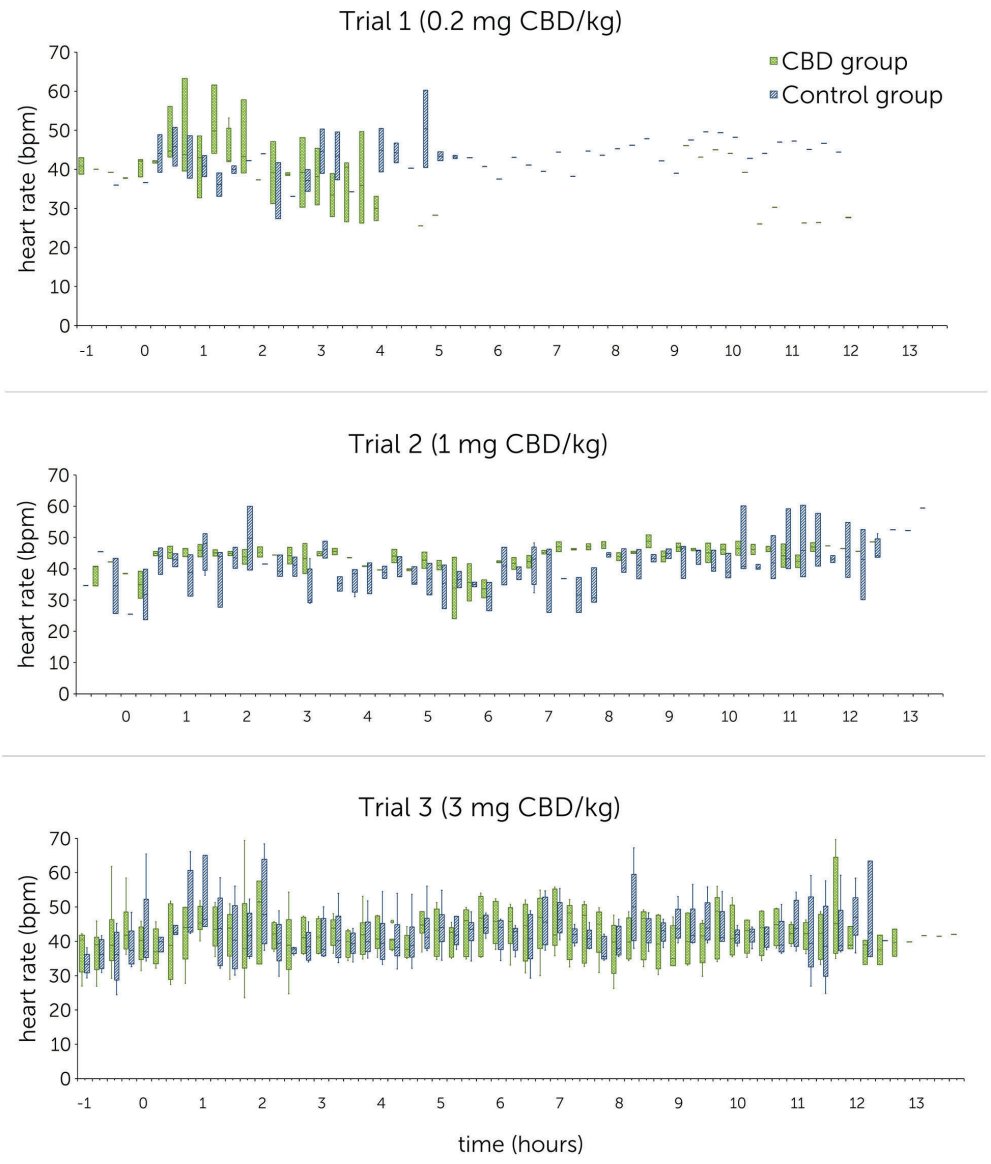

FIGURE 4
Heart rates [beats per minute (bpm)] following single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW) at time point 0, displayed in 15-min sections over 12 h. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

levels can be evaluated based on changes of heart rate and heart rate variability in horses (50–53). A comparatively lower HR and increased HRV values (RMSSD and SDNN) indicate an autonomic shift toward a parasympathetic dominance and therefore a reduction of stress (50, 52, 54). In rodents, one-time intraperitoneally injected CBD (10 mg/kg) has been shown to reduce the increase of HR and blood pressure in a stress inducing and fear conditioning setting, suggesting an anxiolytic effect similar to diazepam (24, 55). Another study identified a modest effect of oral CBD (total dose: 30 mg) on resting HR and HRV in humans (29). The relevance for physiological functions with the shown effect is however questionable and should be evaluated with caution as the study design did not include a control group (29). Other studies in horses and dogs showed no influence of CBD on HR or HRV so far: One study in horses found no significant difference in HR during a novel object test between a treatment group fed 100 mg pelleted CBD (~ 0.2 mg CBD/kg) and a control group (36). In dogs, a treatment and a placebo

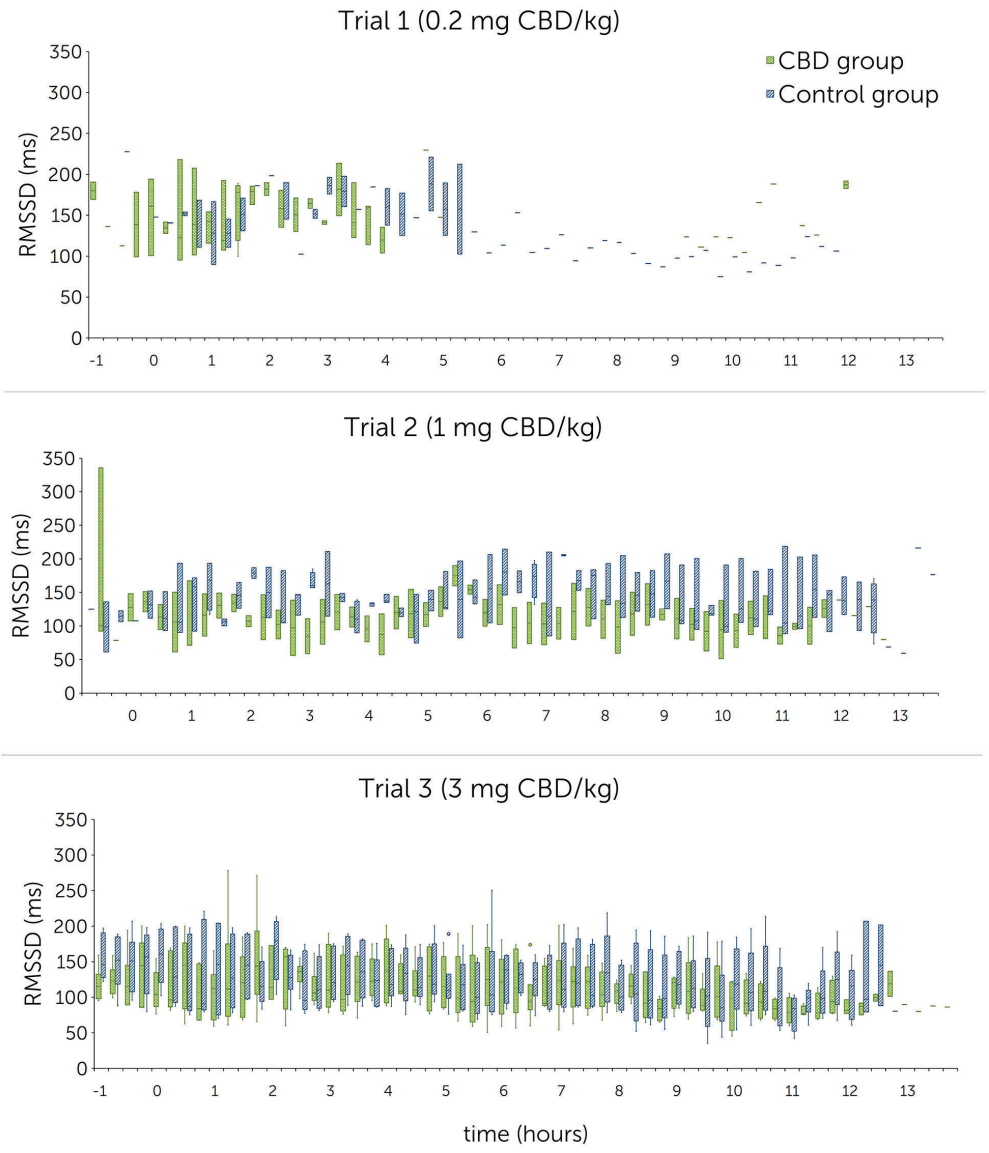

FIGURE 5
Root mean square of successive R-R interval differences (RMSSD) in milliseconds (ms) following single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW) at time point 0, displayed in 15-min sections over 12 h. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

group displayed similar HR and HRV values during a stress test. The dose tested here was 4 mg CBD/kg, administered orally every day over a period of 6 months (56). Similarly, dogs treated orally with 1.4 mg CBD/kg showed no significant changes in RMSSD and SDNN following a fear response test (57). To the best of the authors' knowledge, there are no studies investigating the effect of CBD on resting HR and HRV in healthy horses so far. Due to the short interval of stimulation, it was decided not to specifically analyze HR and HRV during sedation scoring including acoustic and visual stimuli in the current study. HR and HRV compared over the first 2 h after paste administration identified non-significant differences between the treatment and control group in all trials. Comparison within the treatment group showed a consistent increase of the RMSSD compared between all three baseline and trial day values with a significant effect identified for trial 1 (0.2 mg CBD/kg) (Table 7). For SDNN, significant increases were detected for trial 1 and trial 3 (3 mg CBD/kg) (Table 7). These results point toward a decreased sympathetic and an increased

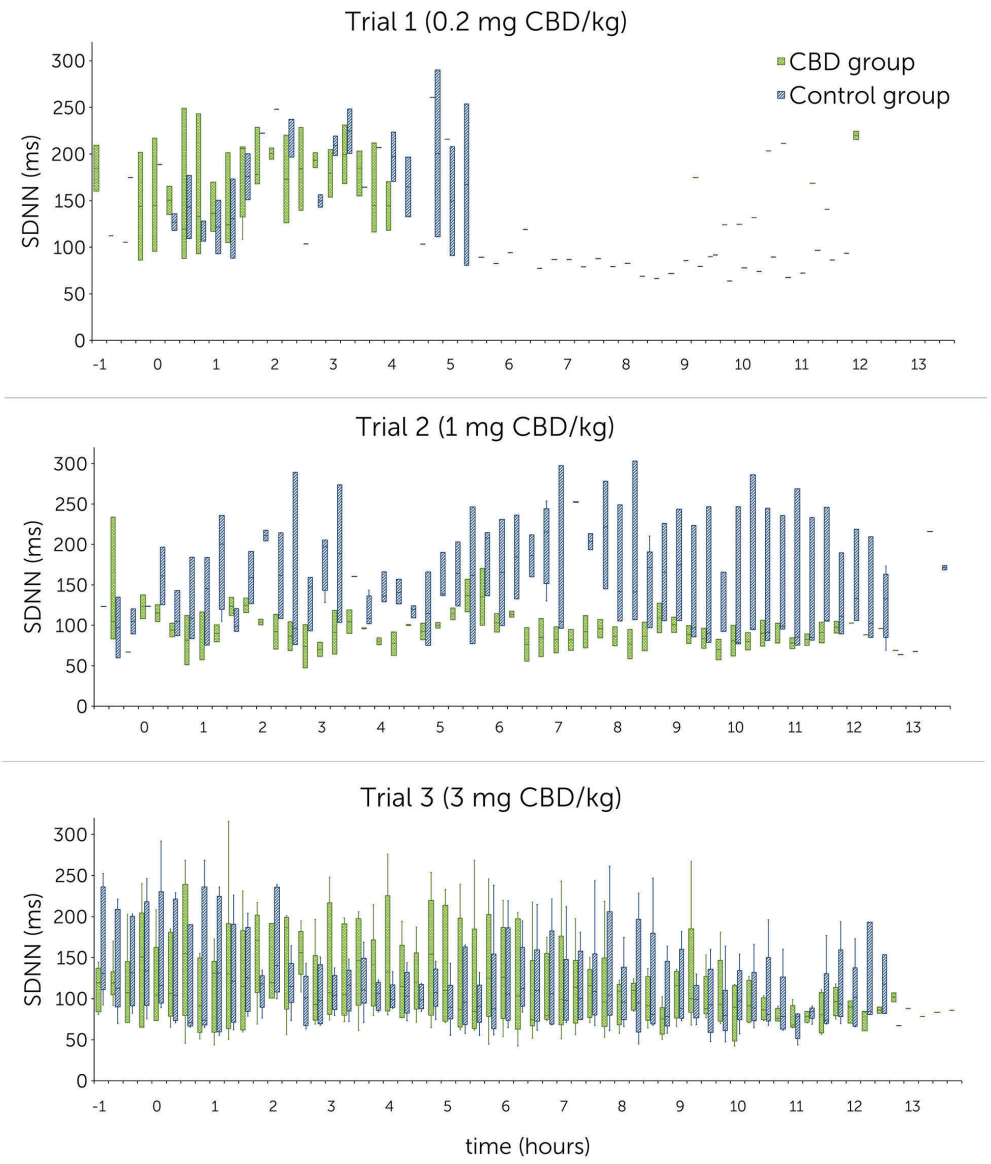

FIGURE 6
Normal-to-normal R-R intervals (SDNN) in milliseconds (ms) following single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW) at time point 0, displayed in 15-min sections over 12 h. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

parasympathetic tonus following CBD administration and support the hypothesized relaxing effect of CBD. However, as the 95% confidence intervals are large, results should still be interpreted with caution.

Cannabis and cannabinoids are FEI declared prohibited substances, with CBD and cannabidiolic acid (CBDA) listed as controlled medication, due to their possible psychotropic and analgesic properties (41). In this study, an influence of CBD in escalating dose levels on equine behavioral parameters could not be confirmed, but it cannot be excluded that higher doses or administration over longer time periods would influence a horse's behavior. As horses in the current study were healthy and displayed a calm behavior throughout, the effect of CBD on stressed or anxious horses would be an additional point of interest.

Limitations of this study include the small sample size and the assessment of single administrations of one CBD containing product only. As horses were closely monitored and sedation levels were scored multiple times per day, a habituation effect

TABLE 6 Fixed effects estimates for comparison between treatment and control group of HR, RMSSD and SDNN values from the first 2 h following single oral administration of cannabidiol (CBD) paste in three escalating doses (0.2mg CBD/kg; 1mg CBD/kg; 3mg CBD/kg).

Parameter	Regression coefficient (β)	95% confidence intervals (CI)	Standard error (SE)	*p*-value
HR (bpm)				
Intercept	43.7	41.4, 46.0	1.1	<0.001
Control group	Reference			
Trial 1 (0.2 mg CBD/kg)	2.6	−1.4, 6.5	2.0	0.196
Trial 2 (1 mg CBD/kg)	0.5	−4.1, 5.1	2.3	0.826
Trial 3 (3 mg CBD/kg)	−1.5	−4.8, 1.8	1.7	0.377
RMSSD (ms)				
Intercept	134.6	123.4, 145.8	5.6	<0.001
Control group	Reference			
Trial 1 (0.2 mg CBD/kg)	11.6	−8.3, 31.6	10.1	0.251
Trial 2 (1 mg CBD/kg)	2.9	−20.5, 26.2	11.8	0.809
Trial 3 (3 mg CBD/kg)	−11.0	−27.5, 5.5	8.3	0.189
SDNN (ms)				
Intercept	135.8	120.7, 150.8	7.5	<0.001
Control group	Reference			
Trial 1 (0.2 mg CBD/kg)	18.1	−8.7, 44.9	13.5	0.184
Trial 2 (1 mg CBD/kg)	−12.1	−43.3, 19.1	15.8	0.445
Trial 3 (3 mg CBD/kg)	−3.8	−26.0, 18.4	11.2	0.734

HR, heart rate; RMSSD, root mean square of successive R-R interval differences; SDNN, standard deviation of normal-to-normal R-R intervals; bpm, beats per minute; ms, milliseconds.

TABLE 7 Fixed effects estimates for comparison within the treatment group of HR, RMSSD and SDNN values from the first 2 h between baseline and following single oral administration of cannabidiol (CBD) paste in three escalating doses (0.2mg CBD/kg; 1mg CBD/kg; 3mg CBD/kg).

Parameter	Regression coefficient (β)	95% confidence intervals (CI)	Standard error (SE)	*p*-value
HR (bpm)				
Intercept	42.5	40.6, 44.4	1.0	<0.001
Baseline values	Reference			
Trial 1 (0.2 mg CBD/kg)	3.4	0.3, 6.6	1.6	0.034
Trial 2 (1 mg CBD/kg)	0.9	−2.7, 4.5	1.8	0.627
Trial 3 (3 mg CBD/kg)	−0.4	−2.9, 2.1	1.3	0.766
RMSSD (ms)				
Intercept	118.4	107.2, 120.5	5.6	<0.001
Baseline values	Reference			
Trial 1 (0.2 mg CBD/kg)	25.0	8.8, 41.1	8.2	0.003
Trial 2 (1 mg CBD/kg)	16.6	−1.8, 35.1	9.3	0.077
Trial 3 (3 mg CBD/kg)	7.7	−5.1, 20.5	6.5	0.233
SDNN (ms)				
Intercept	112.4	99.2, 125.6	6.6	<0.001
Baseline values	Reference			
Trial 1 (0.2 mg CBD/kg)	40.1	20.8, 59.4	9.8	<0.001
Trial 2 (1 mg CBD/kg)	3.0	−19.0, 25.1	11.1	0.785
Trial 3 (3 mg CBD/kg)	21.3	6.0, 36.6	7.7	0.007

HR, heart rate; RMSSD, root mean square of successive R-R interval differences; SDNN, standard deviation of normal-to-normal R-R intervals; bpm, beats per minute; ms, milliseconds.

cannot be excluded. Signs of stress or annoyance as evident on the photographs may partially result from repeated testing. However, as treatment and control groups underwent the exact same protocol, the effect of repeated testing was deemed negligible as it was concluded that it would have occurred similarly in both groups.

5 Conclusions

The analysis of stress parameters did not identify consistently significant effects of orally administered CBD on levels of sedation, the resting heart rate or heart rate variability in horses. Escalating doses (0.2 mg CBD/kg to 3 mg CBD/kg) did not result in a significant reduction of the heart rate, or increased sedation or relaxation. Oral administration of CBD containing paste proved to be well-tolerated and did not cause any side effects. Further research is required to determine specific indications for the use of CBD products in horses.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347–12–2021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FE: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing—original draft. AE: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing—review & editing. KCJ: Formal analysis, Methodology, Software, Validation, Writing—review & editing. NB: Conceptualization, Data curation, Methodology, Project administration, Writing—review & editing. HP: Data curation, Formal analysis, Investigation, Methodology, Writing—review & editing. WB: Conceptualization, Methodology, Project administration, Supervision, Writing—review & editing. CL: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing—review & editing. MW: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The study was funded by the Freie Universität Berlin, the German Equestrian Federation (FN) and the Herosan healthcare GmbH. Herosan healthcare GmbH was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. We acknowledge support by the Open Access Publication Fund of the Freie Universität Berlin.

Acknowledgments

The authors would like to sincerely thank Dr. Marc Machnik and Dr. Ina Schenk for their extensive contributions to this study and Franziska Berger, Julia Bolk, Lena Haas, Katharina Jessat, Anja Kühnel, Dr. Jenny Ries, Clara Schubert, and Elisa Zimmermann for their support with horse handling and laboratory work. We also thank the whole team at the Equine Center Bad Saarow, especially Prof. Dr. Johannes Handler, for providing their facilities and support during the practical part of the study. The authors further acknowledge the support of Conny Pint and Daniela Wimmer and sincerely thank Philine Bank for initiating the project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This study received funding from Herosan healthcare GmbH.

The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare to have full control over the data and no other competing interests.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1305868/full#supplementary-material

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5.3 Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2)

Fabienne Eichler, Anna Ehrle, Marc Machnik, K. Charlotte Jensen, Sabrina Wagner, Natalie Baudisch, Julia Bolk, Magdalena Pötzsch, Mario Thevis, Wolfgang Bäumer, Christoph Lischer, Mechthild Wiegard

Bibliographic Source

Eichler F, Ehrle A, Machnik M, Jensen KC, Wagner S, Baudisch N, Bolk J, Pötzsch M, Thevis M, Bäumer W, Lischer C and Wiegard M (2024): Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2). Front Vet Sci: 10:1305873. https://doi.org/10.3389/fvets.2023.1305873

Contributions of first author

FE was majorly involved in the study design and planning of the project and was responsible for study execution including animal handling and data collection. FE was responsible for study design, planning of the project, study execution including animal handling and data collection. FE performed the formal analysis including statistics of behavioural observations, heart rate and cortisol parameters and wrote the main draft of the manuscript.

Contributions of co-authors

All co-authors contributed to study design, planning, validation and writing of the manuscript. AE contributed to data curation, study execution, formal analysis, methodology, project administration, supervision and validation. SW performed the analysis of cortisol levels under supervision of MT and MM. KCJ supervised the statistical analysis. NB contributed to study execution. JB and MP were involved in data analysis of behavioural parameters under supervision of MW. WB, CL and MW contributed to formal analysis, methodology, project administration, supervision and validation.

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 03 January 2024
DOI 10.3389/fvets.2023.1305873

OPEN ACCESS

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RECEIVED 02 October 2023
ACCEPTED 10 November 2023
PUBLISHED 03 January 2024

CITATION
Eichler F, Ehrle A, Machnik M, Jensen KC, Wagner S, Baudisch N, Bolk J, Pötzsch M, Thevis M, Bäumer W, Lischer C and Wiegard M (2024) Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2). *Front. Vet. Sci.* 10:1305873. doi: 10.3389/fvets.2023.1305873

Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2)

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As a remedy against stress and anxiety, cannabidiol (CBD) products are of increasing interest in veterinary medicine. Limited data is available describing the actual effectiveness of CBD in horses. The aim of this study (part 2 of 2) was to analyze stress parameters via behavioral observation, heart rate monitoring and assessment of blood and saliva cortisol levels in healthy horses treated repeatedly with a CBD containing paste. Twelve horses were randomly assigned to a treatment or a control group. Two pastes were orally administered in a double-blinded study design, one paste containing CBD and one paste without active ingredient. Both pastes were administered twice daily over 15 days (dose: 3 mg CBD/kg). Behavioral observations were conducted daily using a sedation score and a rating of facial expressions, based on the previously described facial sedation scale for horses (FaceSed) and the Horse Grimace Scale. Blood and saliva samples were obtained regularly to determine cortisol levels throughout the study. Cortisol levels were analyzed by means of liquid chromatography/tandem mass spectrometry (LC/MS/MS). Behavioral observations and cortisol levels were compared between groups. Prior to paste administration, a novel object test was performed and the horses' reaction to loading on a trailer was recorded. Both tests were repeated after 13 days of paste application. Movement patterns such as different gaits during the novel object test were evaluated and an ethogram was designed to assess exhibited behavioral traits. Cardiac beat-to-beat (R-R) intervals were recorded throughout and evaluated using heart rate (HR) and heart rate variability (HRV) parameters. Blood and saliva samples for cortisol analysis were taken before and after the tests. Daily behavioral observations and cortisol levels did not differ between the treatment and the control group. Similarly, analysis of movement patterns, HR, HRV and cortisol levels during the novel object test and trailer test did not identify significant differences between the groups. Regularly administered oral CBD (3 mg/kg BID over 15 days) had no statistically significant effect on behavioral observations, cortisol levels, HR and HRV in horses. Further research is required to establish adequate doses and indications for the use of CBD in horses.

KEYWORDS

behavior, CBD, equine, FaceSed, heart rate variability, Horse Grimace Scale, novel object test, sedation score

1 Introduction

Supplements containing cannabis compounds have been promoted as remedies for the treatment of numerous conditions such as anxiety or osteoarthritis in human and animal patients (1–5). Their popularity has increased in recent years but few scientific studies have investigated the actual effectiveness in animals and specifically horses (6–8). The predominant cannabis compounds include the phytocannabinoids cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC), which is known for its psychoactive properties (9–11). CBD is currently under investigation for its proposed relaxing and anxiolytic effects in humans, rodents and dogs (3, 12–23). CBD interacts directly with the serotonin$_{1A}$ (5-HT$_{1A}$) receptor (1, 24–27) and indirectly with the cannabinoid type 1 (CB$_1$) receptor from the endocannabinoid (eCB) system by inhibiting the deactivation of endogenous cannabinoids (28–30). 5-HT$_{1A}$ receptors and the eCB system regulate stress responses and can exhibit an anxiolytic effect when activated (27, 31–33). The CB$_1$ receptor and its significance as a therapeutic target are currently under investigation (34, 35).

The pharmacological activity of the acidic forms of CBD and THC, cannabidiolic acid (CBDA) and Δ^9-tetrahydrocannabinolic acid (THCA), has been scarcely reported so far (9). CBDA and THCA have been shown to interact with the eCB system with their functionality still under study (36–38). In addition to phytocannabinoids, cannabis plants contain terpenoid and flavonoid contents which are described to exhibit multiple effects, including anti-inflammation or sedation (39).

In the European Union (EU), companies declare their cannabis products for horses as "nutritional supplements" as opposed to medicinal products and are therefore not under regulation by the European Medicines Agency (EMA). To date, there is no authorized cannabis veterinary medicinal product in the EU or North America available (40). The Fédération Equestre Internationale (FEI) has banned all cannabis products due to the exhibition of potentially psychotropic effects (41). Since 2022, CBD is classified as a controlled medication (41).

In horses, options for the assessment of stress-responses include behavioral observations such as sedation scores or facial expression scales (42–46) as well as the analysis of physiological parameters like cortisol levels (47–51), heart rate and heart rate variability (48, 52–54). A common and frequently documented test to evaluate stress or fear in animals is the novel object test (6, 54–57). One report has assessed the effect of CBD in horses using a novel object test with evaluation of reactivity and heart rate after daily feeding of CBD pellets (dose: ~0.2 mg CBD/kg SID) for 6 weeks (6). When compared to a control group, reactivity scores were lower, but no significant difference in heart rate was identified (6).

Transportation and loading on trailers cause stress responses in horses which are reflected in increased heart rates and cortisol levels (58–60). Different training methods or even sedatives can be applied to effectively reduce these stress responses (58–61). No report has documented a potential effect of CBD on equine stress levels during loading on a trailer so far.

The aim of this study was to validate equine behavior and stress reactions including the response to a novel object test and a trailer test via heart rate and cortisol level monitoring in healthy horses following repeated oral administration of CBD containing paste (3 mg CBD/kg BID) for 15 days. The authors hypothesized that regular CBD administrations would have a calming effect in horses.

2 Materials and methods

2.1 Animals and study products

Twelve horses (seven mares and five stallions, Haflinger x Warmblood cross) were enrolled in the study. Horses were randomly assigned to a treatment or a control group ($n = 6 + 6$). Horses' age was 3–16 years (median: 11 years) with an average body weight of 488 ± 55 kg in the treatment group. In the control group, the age was 10–26 years (median: 10.5 years) and the body weight 443 ± 56 kg. This study was designed as a prospective, randomized clinical trial. Study products were two pastes for oral administration, one containing 55% full spectrum CBD plant extract, medium-chain triglyceride (MCT) coconut oil, naturally occurring phytocannabinoids, terpenes, flavonoids and beeswax with a THC content of <0.2% (TAMACAN XL 55%®, Herosan healthcare GmbH, Austria). The second paste lacked an active ingredient and contained MCT coconut oil and beeswax [see part 1/2 for further detail (62)]. Pastes were labeled as "A" or "B" to conceal the formulation. The study was approved by the competent authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347-12-2021). Animals included had to pass a general physical examination by a licensed veterinarian and had a blood sample analysis including assessment of a complete blood count (CBC), kidney and liver biomarkers prior to study start. Exclusion criteria included irregularities during examination of the circulatory, respiratory and gastrointestinal systems, and signs of pain or inflammation such as fever and high white blood cell counts.

2.2 Multiple dose study

The multiple dose study started following a wash-out period of 25 days after the dose escalation study (62) to ensure a complete elimination of all cannabinoids following previous CBD applications. The day before study start, horses were physically examined, and a jugular vein catheter was aseptically placed. The jugular vein thrombophlebitis of one mare from the previous study part had resolved by this time (62). Serum and urine samples were tested for residual cannabinoid contents from the previous study part.

Throughout the study, physical examination was repeated daily in every horse. Pastes (dose: 3 mg CBD/kg) were administered before feeding every 12 h (6:30 a.m. and 6:30 p.m.) for 15 days. Equine behavioral observations were video recorded daily between 7:30 am and 8:30 am using two acoustic stimuli (clicker and crackling of a plastic bag) and one visual stimulus (waving of a pink cloth). Video length was between 30 s and 60 s. Photographs of the horses' faces were further taken once daily between 8:30 and 9:30 a.m. for assessment of facial expressions. Analysis of facial expressions was performed on one photo per horse and day. Videos and photographs were taken with an Apple iPhone SE® (Apple Inc., CA, United States). Analysis of facial expressions was based on the facial sedation scale for horses (FaceSed) (43) and the Horse Grimace Scale (45). Facial parameters analyzed included orbital opening, position of ears, tension of chewing muscles represented by their visible presence, relaxation of lips and dilation of nostrils (62). Figure 1 shows a timeline of the study.

Blood and saliva samples obtained for assessment of cannabinoid levels (63) were additionally analyzed for cortisol levels. Samples were taken on the day before start of paste administrations (day 0), days 1–4, 8, 15–19, 23, and 30 (Figure 1). To avoid any influence of the circadian rhythm, only samples taken between 8:00 a.m. and 9:00 a.m. were chosen for cortisol analysis. Per each horse, 10 mL of blood was collected into serum separating tubes, stored at room temperature for 30–60 min and centrifuged at 3,000 × g for 10 min. From each tube, 5 mL of serum was then transferred into a fresh tube to be frozen and stored at −20°C. Samples were analyzed per each individual horse. To further analyze cortisol levels, saliva samples were taken with synthetic swabs (Salivette®, SARSTED AG & Co. KG, Nümbrecht, Germany). Swabs were removed from the tube using Gross-Maier Dressing Forceps and inserted into the horse's mouth for approximately 30 s. Two to three swabs were used for each sample. Salivettes® were centrifuged at 1,000 × g for 10 min. Saliva was subsequently transferred into new tubes, frozen and stored at −20°C.

2.3 Novel object test and trailer test

To obtain baseline behavioral values, a novel object test and horses' reactions to loading on a trailer were video recorded 3 days before the start of paste administration. Blood and saliva samples were taken for measurement of cortisol levels immediately prior to the novel object test. A Polar® H10 heart rate sensor (Polar® Electro Oy, Kempele, Finland) was attached to an electrode belt which spanned around the horse's chest. Each horse's coat was trimmed and moisturized with water over the heart base between the 4th and 5th intercostal space to enhance signal transmission. The heart rate sensor was connected to a mobile device via Bluetooth to record cardiac beat-to-beat (R-R) intervals using the Polar® Equine App (Version 1.2.1, Polar® Electro, Kempele, Finland). For the novel object test, an inflatable pool raft (approximately 170 × 80 × 10 cm, yellow pineapple) served as the unknown object. The pool raft was chosen for its bright and large exterior, and to minimize the possible risk of injury for the animals. The test began with horses being led into a round pen (Ø 15 m). The person leading the horse left the round pen and the object was lowered from the ceiling in the center of the round pen (Figure 2). After 10 min, the horse was taken out of the round pen and the object was raised to the ceiling again.

Each horse was subsequently led into a riding hall, where a trailer was parked. Horses were guided directly toward the trailer and up the ramp. If a horse was not willing to walk up the ramp, it was led back in a circle for another attempt (maximum five attempts). A second person was then asked to stand behind the horse and support its guidance toward the trailer. Loading was not enforced by any additional measures. After the tests, blood and saliva samples were obtained for later assessment of cortisol levels.

Both tests were repeated after 13 days of paste administration (Figure 1), as CBD concentrations in serum were expected to have reached a steady state by this time (63). A new pool raft with similar dimensions but differing outer appearance (green turtle) was chosen for the second novel object test. The remainder of the protocol including the setup for loading on a trailer remained the same. All tests were recorded using a video camera (GoPro HERO10®, San Mateo, United States).

2.3.1 Assessment of novel object test

All video recordings were randomized and blinded. Evaluation was performed by one observer who was experienced in equine behavior studies and not aware of the horses' group assignments. For each recording, the time periods spent in different movement patterns were assessed. Movement patterns included sniffing the ground, standing still, moving in each gait (walk, trot, canter) and rolling.

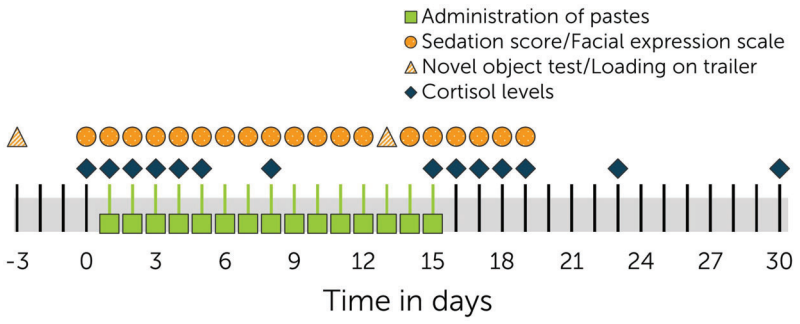

FIGURE 1
Timeline of multiple dose study. Pastes (3 mg CBD/kg and control) were administered twice daily (n = 6 + 6 horses) from days 1 to 15.

FIGURE 2
Novel object test. A pool raft (yellow pineapple) was chosen as the unknown object. The horse is wearing an electrode belt with a heart rate sensor around its chest.

During locomotion in each gait, the number of changes in direction were additionally documented. The horses' reactions to the novel object itself were recorded by taking a note of the time it took a horse to first fixate the object visually, first approach the object and first touch the object.

2.3.2 Assessment of trailer test

Randomized and blinded video recordings were assessed by an observer experienced in equine behavior studies, who was not involved in the previous study parts. Each horse's compliance with entering the trailer was scored on a scale from 0 to 7 for each attempt (Table 1). The attempt with the highest score was selected for statistical analysis.

2.3.3 Ethogram

An adjusted ethogram was developed to evaluate the behavioral traits shown throughout the novel object- and the trailer tests (Table 2). Randomized and blinded video analysis was performed by three observers who were not involved in the previous study parts but specifically trained for equine behavioral assessment. The number of behavioral traits displayed per horse was evaluated. Results of all three assessments were pooled to median values for further analysis.

TABLE 1 Behavioral scoring for trailer test.

Score	
0	Horse stops in front of the ramp
1	One front leg is on the ramp
2	Both front legs are on the ramp (with support)
3	Both front legs are on the ramp (no support)
4	Both front legs are in the trailer (with support)
5	Both front legs are in the trailer (no support)
6	Horse is in the trailer (with support)
7	Horse is in the trailer (no support)

"Support" refers to a second person standing behind the horse to guide it on the trailer.

2.3.4 Assessment of heart rate and heart rate variability

Each cardiac beat-to-beat (R-R) recording was divided into sections of 5 min as previously described (54). Automatic beat correction was applied to remove artifacts (threshold: very low, 0.3 s). Heart rate (HR) and heart rate variability (HRV) including the following parameters: mean HR in beats per minute (bpm), root mean square of successive beat-to-beat differences (RMSSD in milliseconds, ms) and standard deviation of normal-to-normal R-R intervals (SDNN, ms) were evaluated using the software Kubios® HRV Standard (ver. 3.5, Kubios® Oy, Kuopio, Finland).

2.4 Assessment of cortisol levels

Cortisol levels in serum and saliva samples were determined by means of high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS). Information on the sample preparation/extraction, instrumental conditions, validation, analysis and method validation are summarized in the Supplementary material.

2.5 Statistical analysis

Data were recorded in Microsoft Excel® (Version 2304) and statistical analysis was performed with SPSS® Statistics 27 (IBM®, NY, United States). Data were visually inspected and tested with a Shapiro–Wilk test for normal distribution. Behavioral observations (sedation score, facial expression scale) and cortisol concentrations were analyzed using an analysis of variance (ANOVA) with a Greenhouse–Geisser correction and a general linear model for repeated measures to test for differences between the treatment and the control group over time. Cortisol levels in serum and saliva were further tested for correlation using Spearman's rank correlation coefficient.

For the novel object test and the trailer test, the differences between movement patterns, reactions to the unknown objects,

TABLE 2 Ethogram developed for evaluation of the [†]novel object test and [§]trailer test.

Behavioral trait	Description
Bucking[†]	Fast dynamic movement in which the horse lowers its head, rounds its back and jumps in the air, sometimes leaving the ground with all four legs while kicking with the hindquarters
Cocking hindleg[†]	Horse standing firmly on three legs while one hindleg touches the ground with only the tip of the hoof
Defecating[†]	The horse relieving itself from fecal matter
Digging/scratching[†§]	Standing firmly on three legs while purposefully scratching the ground with the tip of one front hoof
Ear movement[§]	(Independent) flickering of one or both ears
Flehmen response[†]	Stretching the neck and the head upwards while curling the nose and exposing the teeth
Freezing[§]	Freezing of the horse with tense posture and forward gaze
Head tossing[†§]	Abrupt, powerful, short movement of the head and neck sideways or upwards; usually combined with tilting of the head
Licking/chewing[†]	Movement of the jaw that results in opening and closing of the mouth including movement of the tongue
Looking around or behind[§]	Turning the head and neck toward the back without leg movements
Neighing[†§]	The sound of a characteristic noise of a horse with different volumes and voice pitches
Remaining near exit[†]	The horse seeks close proximity to the exit of the round pen and remains there
Rolling[†]	Laying on the ground and demonstration a rolling motion, sometimes tilting over to the other side
Sniffing[†]	Horse lowers the head and sniffs the ground
Sniffing the ramp[§]	Horse lowers the head and sniffs the ramp
Snorting[†§]	Accelerated exhale through the nostrils accompanied by a characteristic flapping sound of the nostrils
Stomping[†]	Lifting of one leg and placing it back down forcefully
Tail swishing[†§]	Short, intense, omnidirectional movement of the tail
Treading on the spot[§]	Lifting and lowering the hooves without forward, backward or sideways movements
Urinating[†]	The horse relieving itself from urine in a characteristic stand
Walking backwards[§]	Stepping backwards
Walking sideways[§]	Stepping sideways

scores for loading on a trailer, ethogram behavioral traits and cortisol levels during the first test (baseline) and after 13 days of paste administration were calculated for each horse. Differences between the treatment and control group were compared using a *t*-test (for normally distributed data) or a Mann–Whitney-U-Test (for not normally distributed data). For the ethogram, intraclass correlation coefficients determined the level of agreement between the observers for each observed behavioral trait. HR, RMSSD and SDNN parameters obtained during the second test were analyzed using an ANOVA to test for differences between the treatment and the control group. Residuals were visually inspected for normal distribution. The level of significance was $p < 0.05$.

3 Results

3.1 Animals

Daily physical examinations of all horses did not identify any side effects such as gastrointestinal intolerances associated with paste application. On the day before study start, no residual cannabinoid contents were detected in serum or urine. Regular blood analyses did not identify significant irregularities in CBC, kidney and liver biomarkers (63). CBD concentrations in serum reached a steady state after 2 days of CBD paste administration with a mean maximum serum concentration (C_{max}) of 38.4 ± 8.9 ng/mL (63).

3.2 Behavioral observations

Mean values for sedation scores ranged from 34.0 ± 5.0 (day 3) to 51.7 ± 1.5 (day 19) in the treatment group, and 39.0 ± 1.5 (day 15) to 56.0 ± 2.0 (day 19) in the control group. For the facial expression scale, values ranged from 9.7 ± 2.0 (day 3) to 12.6 ± 2.3 (day 9) in the treatment group, and 10.3 ± 0.8 (day 0) to 13.8 ± 1.1 (day 1) in the control group (Figure 3). On 12 out of 18 days, values for sedation scores were higher in the control group than in the treatment group. Comparison using an ANOVA with a Greenhouse–Geisser correction showed no significant differences between groups for the sedation score [$F(3.0, 11.9) = 2.3$, $p = 0.127$] and the facial expression scale [$F(1.0, 1.0) = 1.5$, $p = 0.435$]. Due to technical difficulties, videos and photographs of day 13 and 14 were not assessable for scoring.

3.3 Morning cortisol levels

Throughout the course of the multiple dose study, cortisol levels in serum were on average 54.7 ± 18.6 ng/mL in the treatment group

and 62.2 ± 19.2 ng/mL in the control group. For saliva, mean cortisol levels were on average 0.40 ± 0.30 ng/mL in the treatment group and 0.63 ± 0.45 ng/mL in the control group (Figure 4). Differences between groups were tested using an ANOVA with a Greenhouse–Geisser correction and were non-significant for cortisol levels in serum [$F(4.1, 37.0) = 1.7$, $p = 0.171$] and in saliva [$F(1.6, 3.2) = 1.0$, $p = 0.442$] over all days. Correlation between serum and saliva cortisol levels was $r_s = 0.53$ ($p < 0.001$).

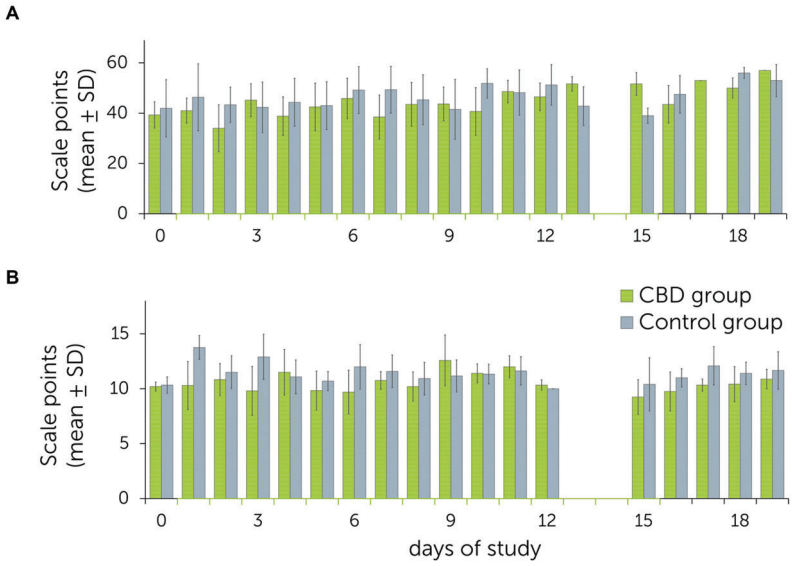

FIGURE 3
Mean ± standard deviations (SD) of behavioral observations obtained during the multiple dose study with daily administration of cannabidiol (CBD) and placebo pastes to a treatment and control group (n = 6 + 6 horses). The treatment group received CBD containing paste from days 1 to 15 (3 mg CBD/kg BID p.o.). **(A)** Summed up sedation scores after acoustic and visual stimulations (clicker, plastic bag, pink cloth). **(B)** Daily facial expression scores. Higher scale points relate to a higher level of relaxation/sedation.

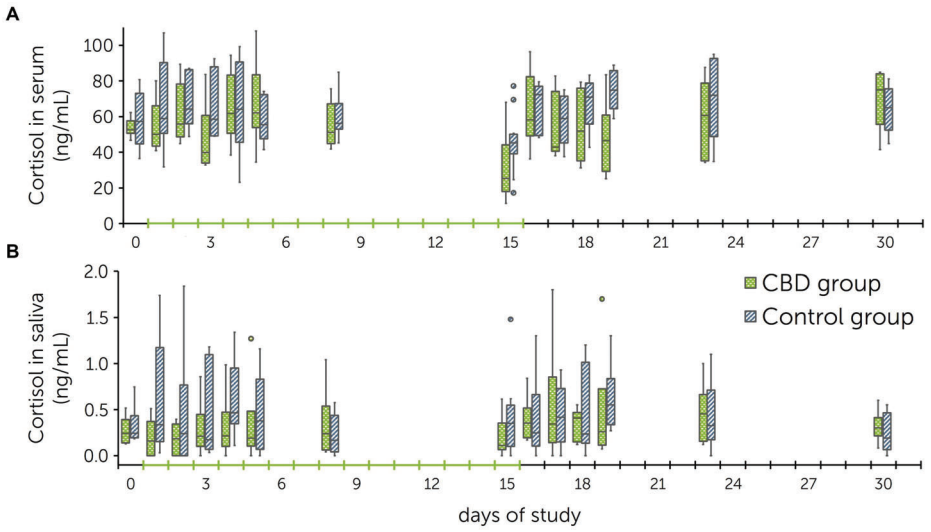

FIGURE 4
Boxplots of cortisol levels in serum **(A)** and saliva **(B)** obtained during the multiple dose study with daily administration of cannabidiol (CBD) and placebo pastes to a treatment and control group (n=6+6 horses). The treatment group received CBD containing paste from days 1 to 15 (3 mg CBD/kg BID p.o.).

3.4 Novel object test and trailer test

3.4.1 Novel object test

The initial reactions to lowering of the pool raft was trotting or galloping alongside the outer parameter of the round pen in all horses. Movements then reduced to walking, standing or sniffing the ground with a subsequent continuation of trotting or galloping in a number of cases. Movement patterns for each individual horse are depicted in Figure 5. The difference between each movement pattern shown during the novel object test before trial start (baseline) and after 13 days of paste administration was calculated for each horse. Comparison of the differences between treatment and control group proved to be non-significant for all movement patterns (sniffing: $p = 0.699$; walking: $p = 0.818$; trotting: $p = 0.818$; galloping: $p = 0.394$; rolling: $p = 0.699$).

During both tests, horses changed direction several times. Differences in the number of changes of direction between before and after treatment ranged from 0 to 4 for each horse in the treatment group and from 1 to 8 for each horse in the control group. There was no significant difference found when compared between groups ($p = 0.485$).

In both novel object tests, all horses first fixated the pool raft visually 1.1–1.4 min after the start with non-significant difference between groups ($p = 0.485$). During the first novel object test (baseline), all horses approached the novel object after approximately 3 min (treatment group: 3.0 ± 1.3 min, control group: 3.0 ± 1.5 min). During the second novel object test, horses in the treatment group first approached the novel object after 4.4 ± 3.4 min and horses in the control group after 1.5 ± 0.5 min. Differences were non-significant ($p = 0.065$). During the baseline novel object test, four horses in each group touched the object. Two horses in the treatment group and four horses in the control group touched the pool raft during the second novel object test. Modes of touching included careful reaching with head and neck, tentative touching, or nibbling. Statistically significant difference was not identified between groups ($p = 0.485$).

3.4.1.1 Novel object test: ethogram

Ten out of fifteen behavioral traits were rated with ICC values of > 0.90. The ICC value for "remaining near exit" was 0.80. "Cocking hindleg" and "stomping" were rated with ICC values between 0.50–0.75, and "licking/chewing" and "snorting" were rated with ICC values < 0.50.

In both groups, the most frequently exhibited trait was "sniffing" (treatment group: median at baseline = 12 times, median after paste administration = 16.5 times; control group: median at baseline = 9.5 times, median after paste administration = 10.5 times). Other behavioral traits (Table 2) were exhibited a median of 0–4 times. Individual stallions showed behavioral traits such as "tail swishing" and "head tossing" up to 18 and 29 times, respectively.

The difference between each behavioral trait exhibited during the baseline test and after paste administration was calculated per horse. Comparison of the differences between groups showed no significant effect [p values ranging from 0.132 ("head tossing") to > 0.999 ("bucking")].

3.4.2 Trailer test

During the baseline test, three horses in the treatment group entered the trailer completely (scores 6 and 7, Table 1), one horse placed both front legs in the trailer (score 4), one horse went as far as putting both front legs on the ramp of the trailer (score 2) and one horse stopped in front of the ramp (score 0). In the control group, two horses entered the trailer (scores 6 and 7), two horses put both front legs in the trailer (scores 4 and 5) and two horses stopped before the ramp (score 0).

After 13 days of paste administration, the scores of six horses (three in each group) did not change (treatment group: scores 7, 7, 0; control group: scores 6, 0, 0). One horse in the treatment group was rated with a higher score (score 2 to 3). Two horses in the treatment group and three horses in the control group scored lower in the second test (treatment group: score 6 to 3, score 4 to 3; control group: score 7 to 6, score 5 to 3, score 4 to 3).

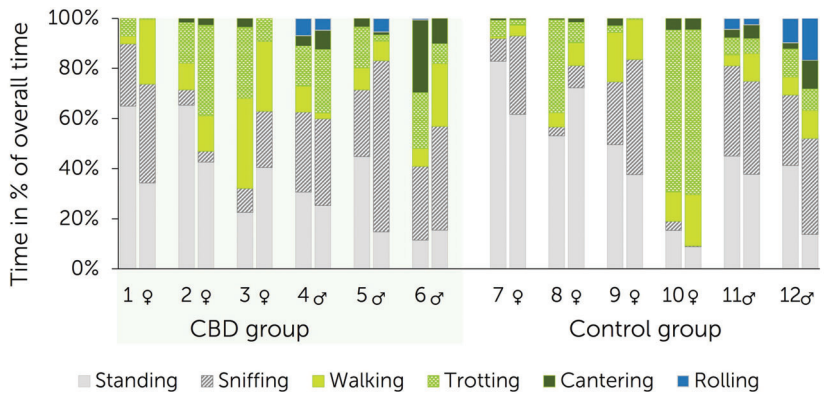

FIGURE 5
Movement patterns during novel object test in direct comparison per individual horse (1–12) between baseline (left bars) and after 13 days of paste administration (right bars) to a treatment and control group ($n = 6 + 6$ horses). The treatment group received a cannabidiol (CBD) containing paste twice daily from days 1 to 15 (3 mg CBD/kg).

For each horse, the differences between scores determined during baseline and after paste administration were calculated with no significant effect when compared between groups ($p = 0.589$).

3.4.2.1 Trailer test: ethogram

Observer agreement using the ICC was rated > 0.90 for six out of twelve behavioral traits. ICC values for "tail swishing," "looking around or behind," and "treading on the spot" were between 0.75 and 0.90. "Ear movement," "freezing" and "snorting" were rated with ICC values of < 0.50.

In both groups, the behavioral trait most frequently observed was "ear movement" during the baseline test (treatment group: median of 5 times; control group: median of 3 times) and after paste administration (both groups: median of 3 times). "Ear movement," "head tossing" and "looking around or behind" was mainly observed in stallions (between 10 and 13 times each). No horse exhibited "digging/scratching." Differences were calculated between the baseline test and after paste administration for each individual horse. Differences were compared between groups using the Mann–Whitney-U-Test with resulting p values ranging from 0.180 ("looking around or behind") to > 0.999 ("digging/scratching," "neighing," "walking sideways").

3.4.3 Heart rate and heart rate variability

Due to technical difficulties, recordings of R-R intervals during the novel object test and the trailer test before study start (baseline) were not available for analysis. It was decided to compare HR and HRV data obtained during the second tests between treatment and control group. The mean values assessed during the novel object test for HR were: 48.6 ± 1.5 bpm, for RMSSD: 93.4 ± 22.1 ms and for SDNN: 87.9 ± 26.3 ms in the treatment group. In the control group, mean values for HR were: 44.9 ± 5.3 bpm, for RMSSD: 113.8 ± 36.5 ms and for SDNN: 113.5 ± 58.9 ms.

During the trailer test, the mean HR was 47.2 ± 3.7 bpm, mean RMSSD was 121.1 ± 21.3 ms and mean SDNN was 118.6 ± 37.6 ms in the treatment group. In the control group, mean values were HR: 46.3 ± 10.7 bpm, RMSSD: 124.2 ± 45.0 ms and SDNN: 132.4 ± 61.0 ms. Analysis using a one-way ANOVA with a Greenhouse–Geisser correction found no statistically significant differences between treatment and control group over both trials for HR: $F(1.5, 12.2) = 1.2$, $p = 0.312$, RMSSD: $F(5, 40) = 1.6$, $p = 0.183$ and SDNN: $F(6, 36) = 1.6$, $p = 0.178$.

3.4.4 Cortisol levels

Serum and saliva samples for cortisol analysis were obtained prior to each novel object test and after each trailer test. Before the first novel object test (baseline), cortisol levels of horses in the treatment group were 44.68 ± 11.08 ng/mL in serum and 0.17 ± 0.09 ng/mL in saliva. After the baseline tests, cortisol levels increased to 68.87 ± 24.95 ng/mL in serum and 0.46 ± 0.38 ng/mL in saliva. Before the second novel object test, serum cortisol levels were 45.22 ± 12.61 ng/mL and saliva cortisol levels 0.15 ± 0.05 ng/mL. After the second trailer test, cortisol levels increased to 47.23 ± 18.27 ng/mL (serum) and 0.35 ± 0.15 ng/mL (saliva) (Figure 6).

Prior to the baseline novel object test, cortisol levels in the control group were 46.28 ± 16.10 ng/mL in serum and 0.26 ± 0.19 ng/mL in saliva. After loading on a trailer, cortisol levels reached 60.87 ± 18.67 ng/mL in serum and 0.20 ± 0.09 ng/mL in saliva. Before the second novel object test, serum cortisol levels were 59.40 ± 25.12 ng/mL and saliva cortisol levels were 0.78 ± 0.48 ng/mL. After the second trailer test, cortisol levels were 61.42 ± 30.30 ng/mL (serum) and 0.50 ± 0.51 ng/mL (saliva) (Figure 6).

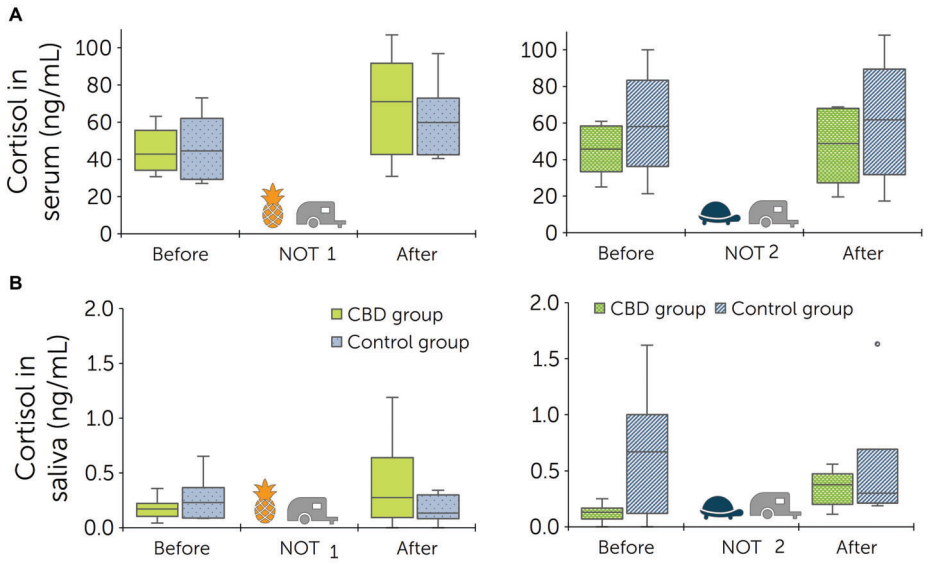

FIGURE 6
Cortisol levels in serum **(A)** and saliva **(B)** before the novel object test (NOT) and trailer test, and immediately after both tests. Tests were performed twice: prior to start of paste administrations (baseline) and following 13 days of paste administrations to a treatment and control group (n = 6 + 6 horses). Pool rafts were used as novel objects [pineapple for the baseline test (NOT 1), turtle for the second test (NOT 2)]. The treatment group received a cannabidiol (CBD) containing paste twice daily from days 1 to 15 (3 mg CBD/kg).

Differences between cortisol levels measured in serum and saliva before and after the tests were calculated for each horse. Comparison of test results from the second tests found a significant difference between groups for cortisol levels in saliva ($p = 0.016$), but not in serum ($p > 0.999$). Within the treatment group, comparison between baseline tests and tests following CBD paste administration showed no significant differences (serum: $p = 0.505$; saliva: $p > 0.999$).

4 Discussion

Regular oral administration of a CBD containing paste at a dose of 3 mg/kg was well-tolerated by all horses in this study. Multiple oral CBD administrations did not have a significant effect on behavioral observations and cortisol monitoring. Parameters investigated in a novel object test and during loading on a trailer did not differ significantly from the control group.

Case reports have described CBD as an effective agent for the treatment of mechanical allodynia, chronic crib-biting and wind-sucking at an oral dose of 0.5 mg CBD/kg BID in horses (64, 65). These reports did not test CBD levels in serum, but previous studies reported maximum CBD concentrations of less than 20 ng/mL in serum following administration of up to 3 mg CBD/kg p.o. (8, 66–71). Two studies found C_{max} levels of 51 ng/mL CBD in serum following oral administration of 2 mg CBD/kg SID for 7 days (67, 70), and C_{max} levels of 55.7 ng/mL CBD in serum following a single oral dose of 10 mg CBD/kg (72). The C_{max} levels of 38.4 ± 8.9 ng/mL in serum reported during the current study (63) are therefore in line with previous reports, and comparatively high (70). In dogs, similar CBD dose levels lead to much higher concentration maxima in serum: one study has shown that the median C_{max} of CBD was 102.3 ng/mL after single oral administration of 2 mg CBD/kg (4). The absorption and retention of CBD in horses seems to be more akin to humans than dogs (70). Single oral intake of 400 mg CBD resulted in a subjective reduction in anxiety in humans with generalized social anxiety disorder (15). However, as no therapeutic serum concentrations for anxiety in humans are available so far, further studies are required to translate administered CBD dose levels to therapeutic serum concentrations.

The facial expression scale used in this study was based on the facial sedation scale for horses (FaceSed) and the Horse Grimace Scale (HGS) (43, 45). Two studies have reported an effective assessment of facial expressions using the HGS to indicate pain levels (73, 74). In the current study, daily behavioral observations of sedation levels using a sedation score and a facial expression scale did not differ significantly between treatment and control group. This assessment is in line with previous studies that found no significant effect on sedation levels following regular CBD pellet feedings (~0.29 mg CBD/kg over 56 days) in horses (7) and oral administration of CBD treats (4.5 mg CBD/kg BID over 21 days) in dogs (18). Reports on US veterinarians and pet owners' perceptions of CBD and hemp use in dogs state that sedation/tiredness were the most commonly observed side effects (75–77). In humans, sedation was reported as a side effect following daily oral intake of 600 mg CBD over 6 weeks (78). As doses were higher in these reports, the question remains whether increased dose levels and therefore increased serum concentrations would lead to a similar effect in horses.

Cortisol is a steroid hormone which is subject to a circadian rhythm. Cortisol levels assessed in previous publications were reported to be highest between 8 am and 12 pm (serum: 25–70 ng/mL; saliva: 0.55–0.70 ng/mL) (50, 79) and are comparable to levels reached in the current study. Depending on the time of day and stress exposure, saliva levels can reach up to 3 ng/mL in horses but usually stay below 1 ng/mL (49, 50, 80). Saliva sampling is a noninvasive, pain-free additional technique to gain more information about cortisol levels (49, 81). Salivary and serum cortisol levels have been reported to have different degrees of correlation ($r_s = 0.32–0.80$) (50, 81). In this study, a moderate correlation was seen between serum and salivary cortisol levels ($r_s = 0.53$) (82). Minor disruptions leading to stress responses can result in deviations from the normal circadian cortisol rhythm and may elevate cortisol levels in blood (50, 79). In this study, no significant effect of CBD on morning cortisol levels was identified.

Novel object tests have been used in a variety of species and can be performed with different unknown objects (54–57) or even unknown horses (Novel horse test) (83). Novel object tests are designed as fear tests and are used to document the intensity of an animal's fearfulness when confronted with the unknown object. As no standard protocol exists, neither regarding the kind of object nor the duration of exposure, scoring of reactions and assessment of additional parameters (such as heart rate) tend to vary. In this study, two novel object tests were performed with similarly sized yet differently colored and shaped objects (pool rafts: yellow pineapple and green turtle) to make the test results comparable and exclude a habituation effect. One report tested habituation to a frightening stimulus (white nylon bag) in 2-year-old colts. It was concluded that the horses were habituated to the stimulus after four training sessions which were all conducted within 1 day (84). As the novel object tests performed in this study were only performed twice and were 16 days apart, habituation was considered to be an unlikely limiting factor. The effect of CBD in horses has been tested in another study using a novel object test following daily oral administration of CBD pellets (~0.2 mg CBD/kg) (6). A significantly lower degree of reactivity compared to a control group was documented (6). A fear response test performed in dogs following oral CBD treatment (1.4 mg CBD/kg) showed no significant effect (85). In agreement with this report, the current study found no significant difference between treatment and control group regarding movement patterns. Reaction times to the novel object differed between groups: during the first novel object test, horses in both groups took about 3 min to first approach the novel object. During the second test, horses in the treatment group took more time to first approach the object (4.4 ± 3.4 min) than horses in the control group (1.5 ± 0.5 min). These differences could suggest that CBD does either not exhibit a fear-reducing effect in the studied dose level, or that CBD has a relaxing effect and reduces the horse's interest in the novel object. Statistical analysis showed that the differences between groups are bordering on significance ($p = 0.065$), which might be biased by the small sample size. Future tests should include larger sample sizes and potentially nervous horses when determining CBD's effect as a fear-reducing or anxiolytic agent.

Loading on a trailer is considered a stressful event for horses (58–60). Different training methods are described to reduce horses' discomfort and anxiety (58–60). In addition to training, sedatives like acepromazine may be used to reduce stress responses (61). Oral CBD (total of 400 mg, single administration) has been reported to subjectively decrease anxiety in humans with generalized social

anxiety disorder (15). The effect of CBD on horses' reactions to loading on a trailer has not been reported yet, but results of this study suggest that it does not increase horses' willingness to enter a trailer at the tested dose level.

Behavioral traits displayed by horses during the novel object- and the trailer test were assessed using a customized ethogram. Behavioral observations may be performed using a software (53) or handwritten lists prepared by one to four independent observers (73, 74, 86). To reduce subjectivity, three observers rated behavioral traits in this study. Most behavioral traits displayed a good (0.75–0.90) to excellent agreement (> 0.90) (87). Behavioral traits with poor agreement (< 0.50) included "ear movement," "freezing," "licking/chewing" and "snorting." Poor scores might be related to an insufficient description of the respective traits, or to the more difficult detection of smaller movements such as "ear movement" or "licking/chewing" especially in combination with other movements when watching a video recording. A wide variety of behavioral traits were assessed including noises ("neighing") and whole body movements ("walking backwards"), as well as behaviors indicative of stress such as "bucking" or "head tossing" (88). No significant differences in displayed behavioral traits were identified between treatment and control group.

Studies investigating heart rate (HR) and heart rate variability (HRV: RMSSD and SDNN) have shown that a decrease in HR and increase in RMSSD and SDNN suggest an autonomic shift toward a parasympathetic dominance and are therefore indicative of the horse's stress levels (48, 54, 89–92). Measurement of HR and HRV is an established tool to evaluate stress responses due to pain or anxiety-inducing events (90, 93–96). Additionally, assessments of HR and HRV have been performed during novel object tests (54–56, 97), and loading on a trailer and subsequent transport (98, 99) in horses. The effect of CBD on HR and HRV has been documented in horses, dogs, humans and rodents with varying results. In horses, HR assessed during a novel object test found no significant effect between a treatment group fed 100 mg pelleted CBD (~0.2 mg CBD/kg) and a control group (6). A stress test performed in dogs similarly found no significant differences in HR and HRV values between a treatment (single oral administration of 4 mg CBD/kg) and a placebo group (100). A second report in dogs equally identified no significant changes in RMSSD and SDNN following a fear response test when treated orally with 1.4 mg CBD/kg (85). In contrast, single intraperitoneal CBD administration in rodents (10 mg CBD/kg) significantly reduced the increase of HR and blood pressure in a stress inducing and fear conditioning setting, suggesting an anxiolytic effect (14, 16). In this study, HR values were higher and RMSSD and SDNN were lower in the treatment than in the control group, indicating a less pronounced parasympathetic state in the treatment group. However, as these differences were statistically non-significant, their relevance is debatable.

Measurement of cortisol concentrations is an established parameter for stress evaluation in horses (49, 51, 81, 92, 99). When comparing the cortisol levels before and after the novel object- and trailer tests, cortisol levels in serum increased to varying degrees (Figure 6). Within the treatment group, the increase was less pronounced after the second round of tests. Statistical analysis showed that this reduction was non-significant. In the control group, salivary cortisol levels had decreased after both test rounds. The difference between treatment and control group was therefore found to be significant ($p = 0.016$). The effect of CBD on cortisol levels has been investigated in humans, dogs and horses with varying results (17, 66, 100–102). After a stress test, dogs that received oral CBD (4 mg CBD/kg) showed significantly lower serum cortisol concentrations than a control group (100). In horses, one study compared cortisol levels between horses that were administered CBD oil and horses receiving olive oil after transportation with no significant findings (66). Studies performed in humans are difficult to compare due to their differing designs and intentions, but have similarly not found a significant effect of CBD on cortisol levels (101, 102).

As all cannabinoids are listed as prohibited substances by the FEI, and CBD is defined as a controlled medication (41), future studies are required to determine what effects oral dosing of CBD exactly exerts in horses, and what dose levels and intervals are needed to achieve these effects. No consistently significant effects on equine behavior were observed in this study.

A small sample size is the main limitation of this study. Further limitations include the missing recordings of R-R intervals during the novel object test and the trailer test before study start (baseline). Consequently, comparison of HR and HRV was carried out between groups following paste administration. Subjects were healthy horses that did not show behavioral problems. Further trials with larger sample sizes are needed to validate the potential effectiveness of CBD in anxious or nervous horses. Future studies may also include more detailed assessments of HRV parameters including the parasympathetic tone activity (PTA) index. Oral dosing using different formulations such as micellar formulation should also be considered (72). Clinical studies as have been performed with dogs (4) are of interest to further assess the potential use of CBD in equine medicine.

5 Conclusion

This study did not detect consistently significant effects of regularly administered oral CBD (3 mg/kg BID over 15 days) on behavioral observations or morning cortisol levels in healthy horses. Horses' reactions to a novel object and loading on a trailer were tested with no significant differences identified between treatment and control group. Parameters assessed included movement patterns, reaction to the novel object, heart rate and heart rate variability, and cortisol levels in serum and saliva. No adverse reactions were observed following multiple administrations of a CBD containing paste. Further research is required to determine adequate indications for the use of CBD products in horses.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the competent authority for licensing and notification procedures for animal experiments (LAVG) in Brandenburg, Germany (AZ: 2347-12-2021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FE: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft. AE: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. MM: Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – review & editing. KCJ: Formal analysis, Methodology, Software, Validation, Writing – review & editing. SW: Formal analysis, Methodology, Software, Validation, Writing – review & editing. NB: Conceptualization, Data curation, Methodology, Project administration, Writing – review & editing. JB: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. MP: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MT: Methodology, Supervision, Writing – review & editing. WB: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. CL: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. MW: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The study was funded by the Freie Universität Berlin, the German Equestrian Federation (FN) and Herosan healthcare GmbH. Herosan healthcare GmbH was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. We acknowledge support by the Open Access Publication Fund of the Freie Universität Berlin.

Acknowledgments

The authors would like to sincerely thank Hannah Petersen and Dr. Ina Schenk for their extensive contributions to this study, and Franziska Berger, Lena Haas, Katharina Jessat, Anja Kühnel, Dr. Jenny Ries, Clara Schubert, and Elisa Zimmermann for their support with horse handling. We also thank the whole team at the Equine Center Bad Saarow, especially Prof. Dr. Johannes Handler, for providing the horses and their support during the practical part of the study. The authors further acknowledge the support of Conny Pint and Daniela Wimmer, and sincerely thank Philine Bank for initiating the project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1305873/full#supplementary-material

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6 DISCUSSION

6.1 Pharmacokinetic analysis

This study was the first evaluation of the pharmacokinetic properties of CBD in horses following oral administrations over two weeks (3 mg/kg BID). Administration of the CBD paste was well-tolerated and no adverse reactions were observed. Pharmacokinetic analyses were performed using non-compartmental analysis (NCA), and by building a three-compartment model with zero-order absorption utilizing nonlinear mixed-effects modelling (NLME). A model with similar characteristics (three-compartment and zero-order absorption) was chosen in a previous report on CBD PK in horses (Sánchez de Medina et al. 2023). Developing the model added the benefit of combining data from both the dose escalation and the multiple dose study, therefore enhancing the accuracy of estimations for clearance and volumes of distribution. Furthermore, this analysis is particularly suited for study designs involving small sample sizes (Cascone et al. 2013).

In the dose escalation study, NCA indicated a rapid increase in CBD serum concentrations with C_{max} and t_{max} values (12.2 ng/mL at 1.0 h p.a. following single oral administration of 3 mg CBD/kg) comparable to other studies (Table 1). During the multiple dose study, a steady state was reached within 48 hours of CBD administrations. The mean C_{max} over the following days was 38.4 ± 8.9 ng/mL.

As CBD was only administered orally, bioavailability was not assessed in this study.

Values for volumes of distribution over F were V_1/F: 77.1 L/kg, V_2/F: 313.2 L/kg and V_3/F: 242.0 L/kg. These values are considerably higher than in previous reports where CBD was only administered as a single dose (Sánchez de Medina et al. 2023; Yocom et al. 2022) or once daily over seven days (Williams et al. 2022). As CBD was administered twice daily over two weeks in this study, the higher accumulation in peripheral tissues is to be expected.

The findings in this study regarding CBD metabolism are in agreement with a previous report (Ryan et al. 2021), as large amounts of metabolites were identified in serum (7-COOH-CBD, C_{max}: 1251.5 ± 254.0 ng/mL) and urine (7-OH-CBD).

Clearance was estimated at Cl/F: 10.8 L/h/kg which is lower than the results from other equine studies with Cl/F: 45.7 L/h/kg and 15.7 L/h/kg, both following administration of 1 mg CBD/kg (Ryan et al. 2021; Yocom et al. 2022).

Terminal half-life was 161.3 h, considerably longer than previously reported (Table 1). As the rate of elimination is dependent on clearance, a long terminal half-life relates to a comparatively smaller clearance. It is important to note that terminal half-life is highly influenced by study design and choice of analysis for the serum concentration curve: In this

study, the elimination phase was tracked over a comparatively long period (132 h to 360 h after last CBD administration) when the curve had flattened considerably and reached a pseudo-equilibrium state (Toutain and Bousquet-Mélou 2004c).

The results of the current study, as demonstrated by the NLME model, affirm the previously reported rapid absorption, substantial metabolism, extended retention in peripheral tissues and prolonged elimination phase of CBD (Perucca and Bialer 2020; Ryan et al. 2021; Sánchez de Medina et al. 2023).

6.2 Behavioural observations

To assess a potential relaxing or sedative effect of CBD, the horses' facial expressions were photographed and their reactions to three stimuli (two acoustic and one visual) were video recorded. Photographs and videos were analysed using specifically developed scales to allow for a detailed evaluation of the observed reactions. The scales were based on the facial sedation scale (FaceSed) for horses (Oliveira et al. 2021), the Horse Grimace Scale (Dalla Costa et al. 2014), and the sedation score by Poller et al. (2013). The analysis of facial expressions and reactions to stimuli did not identify significant differences when compared between horses treated with CBD and a control group, neither during the dose escalation study nor the multiple dose study.

During the dose escalation study, the heart rate was similarly unaffected in the first two hours following CBD application when compared between groups. Comparison of heart rate variability values within the treatment group showed a significant increase in RMSSD and SDNN values between baseline and following oral application of a CBD paste. These findings suggest an increase in parasympathetic and decrease in sympathetic activity following CBD administration, potentially supporting a stress-relieving effect of CBD. Nonetheless, given the wide 95% confidence intervals, these results should be interpreted with caution.

During the multiple dose study, differences between morning cortisol levels were non-significant between groups.

Similarly, very few significant differences between treatment and control group were found during the novel object test and trailer test regarding movement patterns, reactivity, behavioural traits defined in an ethogram, HR, HRV and cortisol levels. One noteworthy observation was made when analysing the time to first approach the novel object: Horses in the CBD group took 3 minutes longer to first approach the novel object than horses in the control group, bordering on a significant effect ($p = 0.065$). CBD may therefore reduce a horse's curiosity towards the novel object by acting as a relaxing agent.

In summary, only a few significant effects were identified regarding the influence of CBD on stress levels and sedation in horses this study. The majority of results did not differ significantly between CBD and control group, suggesting that regular CBD administration at 3 mg/kg BID does not affect horse's stress levels and does not have a significant sedating effect.

6.3 Effects of CBD in relation to pharmacokinetics

Previous studies on the effect of CBD on behavioural and stress parameters have tested various dosing regimens with mixed results. In animal studies, CBD doses ranged up to 4.5 mg CBD/kg, while in human studies, doses of up to 50 mg CBD/kg have been tested. A tendency that higher CBD doses may lead to more effective outcomes was observed (Millar et al. 2019). To gain a better understanding of this observation, it is essential to not only describe effective CBD dosing amounts, but also to conduct a comprehensive assessment of the related pharmacokinetics, considering crucial parameters such as absorption rate and clearance (Schwark and Wakshlag 2023; Toutain 2002). In doing so, several considerations must be addressed: The absorption of an orally administered drug into the bloodstream is greatly influenced by the drug's formulation (as exemplified by the differences in C_{max} following CBD administration in micellar or oil formulation, see chapter 2.1.1). Furthermore, the absorption of CBD is marked by a low oral bioavailability, ranging from 6% to 19% across all species (Lim et al. 2020; Perucca and Bialer 2020; Samara et al. 1988; Sánchez de Medina et al. 2023; Turner et al. 2022). Drugs with low bioavailability exhibit significant inter-individual variability in terms of absorption processes, leading to a lack of reproducibility of the clinical efficacy (Toutain and Bousquet-Mélou 2004a). Moreover, it cannot be assumed that similar doses lead to the same serum concentrations in all species. Comparisons between pharmacokinetics in different species (e.g., horses and dogs) should be made with caution: The higher liver capacity in herbivorous species predisposes to a comparatively greater metabolism of lipophilic drugs, therefore resulting in a generally lower bioavailability of such drugs compared to carnivorous species (Baggot and Brown 1998; Martinez et al. 2002). Also, clearance is subject to many inter-species variabilities and is generally higher in herbivorous than in carnivorous species (Toutain et al. 2010).

When all these factors are taken into account, establishing effective CBD doses becomes a challenging task. To the best of the author's knowledge, there is no study investigating the effect of CBD on behaviour, stress or anxiety which also details pharmacokinetic parameters in any species. To nonetheless gain a general idea about what range effective CBD doses and relating pharmacokinetics encompass, the existing behavioural and pharmacokinetic studies

are compared in the following section. CBD doses and resulting effects are put in relation to maximum serum concentrations (C_{max}) as a pharmacokinetic parameter.

In horses, one study has reported a significant pain reduction in patients with mild osteoarthritis treated with phenylbutazone and CBD (0.03 mg/kg SID p.o. over 14 days), compared to horses treated only with phenylbutazone (Interlandi et al. 2024). Pain assessment was performed using the Horse Chronic Pain Scale (HCPS) and physiological parameters. Total HCPS scores were significantly reduced in the CBD group compared to the control group on days 9-14. CBD serum concentrations were not tested in this study (Interlandi et al. 2024).

Regarding behavioural parameters, no controlled study has so far reported a majorly significant effect of CBD on horses (Draeger et al. 2021; St Blanc et al. 2022). The existing studies have only reported dosing amounts (~ 0.2 mg/kg, respectively ~ 0.3 mg/kg SID p.o. over 6, resp. 8 weeks), not CBD serum concentrations (Draeger et al. 2021; St Blanc et al. 2022). In the current report, behavioural tests and evaluation of stress parameters have similarly not shown consistently significant results. Here, the mean C_{max} measured after oral CBD administration at a dose of 3 mg/kg BID p.o. over two weeks was 38.4 ng/mL in serum. As the doses in previous reports are substantially lower than in the current report, it can be assumed that the resulting CBD serum concentrations would have also been lower.

In humans, effective doses for treatment of anxiety and stress have been reported at 300-600 mg (~ 4-9 mg CBD/kg) (Appiah-Kusi et al. 2020; Bergamaschi et al. 2011; Crippa et al. 2011; Faria et al. 2020; Zuardi et al. 1993). Manini et al. (2015) have measured C_{max} values of 181.2 ng/mL to 221.1 ng/mL following oral application of 400-800 mg CBD (~ 5.8-11.4 mg CBD/kg). In dogs, Hunt et al. (2023) reported a significant decrease in stress and cortisol levels following single oral CBD application at 4.0 mg/kg. CBD serum concentrations were not assessed in this study. Pharmacokinetic studies in dogs have reported C_{max} ranging from 102 ng/mL to 268 ng/mL following oral application of 1.0 mg CBD/kg (Gamble et al. 2018; Tittle et al. 2022; Wakshlag et al. 2020). It can be assumed that the resulting CBD serum concentration in the study by Hunt et al. (2023) falls within the same range or potentially higher. However, there are studies for both humans and dogs testing CBD in the same dose range as described above (≥ 4 mg CBD/kg) which have found no significant effect on stress parameters (Mongeau-Pérusse et al. 2022; Morris et al. 2021).

When comparing these studies regarding effective CBD doses and C_{max} values, it is important to note that such comparisons should only be made to obtain a very general understanding of effective CBD serum concentrations. It is exemplified that similar CBD dose ranges lead to higher serum concentrations in humans and especially dogs than in horses. Therefore, to achieve a measurable effect on equine behaviour, higher CBD serum

concentrations are required which are obtained by administering higher doses of CBD than those applied in the current report (3 mg CBD/kg BID p.o., resulting in a mean C_{max} of 38.4 ng/mL). It may be possible that an analgetic effect of CBD in horses can already be achieved following lower CBD dosing as exemplified in one report (Interlandi et al. 2024). When testing higher doses for an effect on behaviour, special attention should be paid to the tolerability of CBD in horses. To date, clinical studies across all species have reported either no or mild side effects. However, side effects tend to have a higher incidence in studies conducted in higher dose ranges (see chapter 3.4).

In summary, future research exploring the effect of CBD on behaviour and stress, should also include assessments of pharmacokinetic parameters to gain a better understanding of effective dose ranges.

6.4 Implications for medication control

To establish irrelevant drug concentrations for medication control in equestrian sports, three parameters must be specified: 1. Effective standard dose per dosing interval of the drug, 2. Bioavailability (F), 3. Plasma clearance (Cl) per dosing interval and 4. Steady state urine to plasma concentration ratio of the drug (Rss) (Toutain and Lassourd 2002). These parameters enable the calculation of the effective plasma concentration and subsequent irrelevant plasma and urine concentrations (IPC and IUC) of a drug, along with the resulting withdrawal times. Bioavailability of orally administered CBD has been reported at 8% to 14% in previous studies (Sánchez de Medina et al. 2023; Turner et al. 2022). In the current report, clearance (Cl/F: 10.8 L/h/kg) and ratio at steady state (Rss: 4.5) were specified. However, a standard dose and resulting EPC could not be established as no effect on behavioural or stress parameters was observed. The calculation of IPC and IUC could therefore not be completed.

Practical conclusions for horse owners can nevertheless be drawn from this study. It was exemplified that CBD has a prolonged terminal half-life of 161.3 hours following application at 3 mg/kg BID p.o. over two weeks. Presumably, this extended half-life is attributed to a high drug distribution into peripheral tissues, resulting in slow elimination. Following the final CBD application, traces of CBD and metabolites were still detected in serum fifteen days post-treatment termination (CBD: 0.5 ng/mL; 7-COOH-CBD: 7.3 ng/mL) and in urine nineteen days post-treatment termination (CBD: 3.5 ng/mL; 7-OH-CBD: 53.3 ng/mL). Horse owners using CBD products for their equine athletes should therefore be made aware of the extended terminal half-life and the potential for detecting traces of CBD and its metabolites over a prolonged period.

When considering the classification of CBD as a prohibited medication in equestrian sports, it is worth looking at regulations in professional human sports. For human athletes, the

World Anti-Doping Agency (WADA) has established that all cannabinoids are prohibited in sports competition, with one exception: CBD. CBD has been excluded from WADA's Prohibited List since 2018 as it does not target the same receptors in the brain as THC (Mareck et al. 2021). WADA regards CBD as neither performance-enhancing nor performance-worsening, permitting human athletes to use CBD freely before competition. The primary factor for the prohibition of cannabis and all cannabinoids is THC. The main target analyte for the detection of possible doping is the THC metabolite 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), with a urinary threshold of 150 ng/mL.

Classifying a drug as a prohibited substance at equine sports events is aimed at ensuring a fair competition, but also at safeguarding the horse from potential harm. The potency of CBD and resulting potential doping-relevant effects remain unclear. Extensive research is required to further investigate the effects of CBD with corresponding serum and urine concentrations in horses, and to establishing appropriate thresholds in serum and urine. Although WADA does not recognize CBD as relevant for doping in human sports, this assessment should not automatically be applied to horses without further research. Until comprehensive studies provide more clarity, a preliminary general prohibition appears to be the most appropriate approach in classifying CBD and all cannabinoids in equestrian sports events.

6.5 Conclusion and outlook

This is the first study considering both the pharmacokinetics and the effects on behaviour and stress parameters following oral CBD administration in healthy horses. The pharmacokinetic analysis showed an extensive metabolism of CBD and implies an extended retention in peripheral tissues, leading to a prolonged elimination phase of CBD and CBD metabolites. Assessment was performed using non-compartmental analysis and a three-compartment model with zero-order absorption, utilizing data from the dose escalation and the multiple dose study. Behavioural observations and analysis of stress parameters did not result in consistently significant effects when compared to a control group. Further research is needed to confirm whether CBD does indeed influence behavioural and stress parameters in horses, as claimed by product manufacturers. Also, CBD products are advertised as an effective management of chronic pain in osteoarthritic equine patients. To date, only one study has investigated the effects of CBD on horses with osteoarthritis, reporting a significant reduction in pain following the administration of both phenylbutazone and CBD (Interlandi et al. 2024). Future studies might involve clinical trials to examine the impact of CBD on horses prone to nervousness and stress, or on pain management in horses with osteoarthritis. If such responses are explored, subsequent pharmacokinetic variables should be determined to establish effective CBD doses and dosing regimens. Single CBD dosing of 10 mg/kg proved

to be well tolerated and may be used as a starting point in upcoming studies (Sánchez de Medina et al. 2023). When testing higher doses, it is important to be attentive to possible side effects, particularly since higher doses have been associated with a greater occurrence of adverse reactions (Millar et al. 2019). Further research should also investigate the possible activity and effect of CBD metabolites in horses: Findings from an in vitro study found that CBD and its metabolites have exhibited toxicity towards human stem cells at high concentrations (Latham et al. 2023). In addition to efficacy and safety considerations, attention should be directed towards the pricing of CBD products, which can vary significantly across different forms such as oils, pastes, and pellets. Products with certified CBD content typically command higher prices. In the context of this study, the paste utilized, "TAMACAN XL® - 5 ml", is priced at €179.90 (Herosan healthcare GmbH, Austria 2024). Administering a single dose of 3 mg CBD/kg to a 500 kg horse therefore amounts to €107.94, and a 10 mg/kg dose would total €359.80. Researchers and practicing veterinarians should carefully consider the anticipated costs before initiating a study or advising horse owners on CBD usage. Until then, the administration of CBD products to sport horses should be approached with caution due to the extended half-life and unclear effect of CBD on behaviour and pain responses.

7 SUMMARY

The use of cannabidiol (CBD) products is becoming increasingly popular among animal owners and veterinarians as an alternative treatment for stress, anxiety or pain in horses. In equestrian sports, all cannabinoids are banned due to their potentially psychotropic effects. However, there are only a few studies on the detection times of CBD concentrations in blood or urine, and the actual effectiveness in horses. The aim of this study was to determine the pharmacokinetic properties of CBD after oral administration in healthy horses and to analyse stress parameters, including behavioural observations, heart rate and cortisol levels.

Study products were two pastes for oral administration, one with CBD as active ingredient and one without active ingredient. Paste administration was blinded. In the first study part (dose escalation study), the pastes were administered in escalating trials as single doses (0.2 mg CBD/kg, 1 mg CBD/kg, 3 mg CBD/kg) to a treatment and a control group. In the second part of the study (multiple dose study), both pastes were administered twice daily for 15 days (treatment group: 3 mg CBD/kg).

For the pharmacokinetic analysis, blood and urine samples were taken daily during both study parts. After day 15 of the multiple dose study, additional samples were collected for two weeks to analyse the elimination phase. Concentrations of CBD, CBD metabolites and other cannabinoids were determined using gas chromatography/tandem mass spectrometry. Pharmacokinetic parameters were assessed using two approaches: Non-compartmental analysis and population pharmacokinetic analysis using a nonlinear mixed-effects model. During the elimination phase, the ratio between the steady-state concentrations of CBD in urine to serum (Rss) was calculated.

In the dose escalation study, behavioural parameters were assessed using photographs to evaluate the horses' facial expressions on a specifically developed scale, which was based on existing scales (FaceSed and Horse Grimace Scale). To identify potential sedation, the horses' reactions to acoustic and visual stimuli were video recorded. The evaluation of the photos and videos was conducted in a blinded manner. In addition, the heart rate was continuously recorded via heart rate sensors throughout the study with subsequent analysis of heart rate (HR) and heart rate variability (HRV).

In the multiple dose study, facial expressions and the depth of sedation were analysed daily following the same protocol as in the dose escalation study. In addition, blood and saliva samples were daily collected and analysed for cortisol levels using liquid chromatography/tandem mass spectrometry. The behavioural observations and cortisol levels were compared between the groups. A novel object test and a trailer test were performed prior to study start. Both tests were repeated on study day 13. Assessment included reactivity,

movement patterns such as gait changes and behavioural characteristics. Heart rate was recorded during the tests and analysed using HR and HRV parameters. Blood and saliva samples were obtained before and after the tests for cortisol analysis.

The CBD paste was well-tolerated and no side effects were observed. The non-compartmental analysis showed a maximum serum concentration of 12.2 ng/ml after single administration of CBD (3 mg/kg). The population pharmacokinetic analysis showed that a three-compartment model with zero-order absorption most accurately describes the pharmacokinetic properties of CBD. High volumes of distribution into peripheral compartments and high concentrations of the metabolite 7-carboxy-CBD were identified. In the multiple dose study, the mean maximum serum concentration was 38.4 ng/mL. The terminal half-life was 161.3 hours in serum and Rss was 4.5.

In the dose escalation study, analysis of behavioural parameters, HR and HRV showed no consistently significant differences between the treatment and control group. During the multiple dose study, daily behavioural observations and cortisol levels also did not differ between treatment and control group. When analysing reactivity, movement patterns, HR, HRV and cortisol levels during the novel object test and the trailer test, no consistently significant differences were observed between groups.

This study was the first to investigate pharmacokinetic parameters combined with the effect of CBD on behaviour and stress after regular oral administration of CBD in horses over two weeks. The pharmacokinetic analysis showed an extensive metabolism of CBD with a high distribution into peripheral tissues and a long elimination phase. The results of the behavioural assessments provided no reliable evidence for a stress-reducing or sedative effect of CBD in horses after regular oral administration at a dose of 3 mg/kg twice daily. The main limitation of this study is the small sample size. Further investigation of the potential stress-reducing effects of CBD in conjunction with pharmacokinetic analysis is essential to determine relevant CBD concentrations for medication control at equestrian sport events. Subsequent studies may consider administering higher CBD doses, such as 10 mg CBD/kg, and specifically explore the effect on horses known to exhibit signs of nervousness and are easily stressed.

8 ZUSAMMENFASSUNG

Orale Cannabidiolgaben beim Pferd: Pharmakokinetische Modellierung, Verhaltensbeobachtungen und Bedeutung für Dopingkontrollen

Cannabidiol (CBD)-Produkte werden bei Tierhalterinnen und Tierhaltern, sowie in der Tiermedizin als alternatives Mittel zur Behandlung von Stress, Angststörungen oder Schmerzen bei Pferden immer beliebter. Im Pferdesport sind alle Cannabinoide aufgrund ihrer potenziell psychotropen Wirkung verboten. Es gibt jedoch nur wenige Studien zu Nachweiszeiten von CBD-Konzentrationen in Blut oder Urin und zur eigentlichen Effektivität von CBD beim Pferd. Ziel dieser Studie ist daher die Bestimmung der pharmakokinetischen Eigenschaften von CBD nach oraler Verabreichung bei gesunden Pferden und die Analyse von Stressparametern, einschließlich Verhaltensbeobachtungen, Herzfrequenz und Cortisolspiegel.

Studienprodukte waren zwei Pasten zur oralen Verabreichung, eine mit dem Wirkstoff CBD und eine ohne aktiven Wirkstoff. Die Verabreichung der Pasten erfolgte verblindet. Im ersten Studienteil (*dose escalation study*) wurden die Pasten in drei aufeinanderfolgenden Versuchen als Einzeldosen an eine Behandlungsgruppe (0,2 mg CBD/kg, 1 mg CBD/kg, 3 mg CBD/kg) und eine Kontrollgruppe verabreicht. Im zweiten Studienteil (*multiple dose study*) wurden beide Pasten zweimal täglich über 15 Tage (Behandlungsgruppe: 3 mg CBD/kg) gegeben.

Für die pharmakokinetische Analyse wurden in beiden Studienteilen täglich mehrere Blut- und Urinproben entnommen. Nach Tag 15 des zweiten Studienteils wurden zwei Wochen lang zusätzliche Proben zur Analyse der Eliminationsphase gesammelt. Die Konzentrationen von CBD, CBD-Metaboliten und weiteren Cannabinoiden wurden mittels Gaschromatographie/ Tandem-Massenspektrometrie ermittelt. Die pharmakokinetischen Parameter wurden anhand von zwei Ansätzen bewertet: Eine nicht-kompartimentelle Analyse und eine populationspharmakokinetische Analyse unter Verwendung eines nicht-linearen gemischten Kompartimentmodells. Während der Eliminationsphase wurde das Verhältnis zwischen der Gleichgewichtskonzentration von CBD im Urin zu Serum (Rss) berechnet.

Im ersten Studienteil wurden Verhaltensparameter anhand von Fotos bewertet, um die Gesichtsausdrücke der Pferde auf einer eigens entwickelten Skala, basierend auf den existierenden Skalen FaceSed und Horse Grimace Scale, zu beurteilen. Zur Bewertung einer potenziellen Sedation wurden die Reaktionen der Pferde auf akustische und visuelle Reize per Video aufgenommen. Die Evaluation der Fotos und Videos wurde verblindet durchgeführt. Während des gesamten Versuchs wurde zudem die Herzfrequenz über

Herzfrequenzsensoren kontinuierlich aufgezeichnet, und anschließend die Herzfrequenz (HR) sowie die Herzfrequenzvariabilität (HRV) analysiert.

Im zweiten Studienteil wurde die Bewertung der Gesichtsausdrücke und der Sedationstiefe gleich dem ersten Studienteil täglich durchgeführt. Zudem wurden jeden Tag Blut- und Speichelproben entnommen und mittels Flüssigchromatographie/Tandem-Massenspektrometrie auf den Cortisolspiegel untersucht. Die Verhaltensbeobachtungen und Cortisolspiegel wurden zwischen den Gruppen verglichen. Vor Studienbeginn wurden ein *Novel Object Test* und ein Anhängertest durchgeführt. Beide Tests wurden an Studientag 13 wiederholt. Zur Auswertung der Tests wurden die Reaktionen der Pferde, Bewegungsmuster wie Gangartenwechsel, und Verhaltensmerkmale anhand eines Ethogramms bewertet. Die Herzfrequenz wurde während der Tests aufgezeichnet und über HR- und HRV-Parameter ausgewertet. Vor und nach den Tests wurden Blut- und Speichelproben für die Cortisolanalyse entnommen.

Die CBD-Paste wurde gut vertragen und es wurden keine Nebenwirkungen beobachtet. Die nicht-kompartimentelle Analyse ergab eine maximale Serumkonzentration von 12,2 ng/ml nach einmaliger Verabreichung von CBD (3 mg/kg). Die populationspharmakokinetische Analyse zeigte, dass ein Drei-Kompartiment-Modell mit Absorption nullter Ordnung die pharmakokinetischen Eigenschaften von CBD am genauesten beschreibt. Es wurden hohe Verteilungsvolumina in die peripheren Kompartimente sowie hohe Konzentrationen des Metaboliten 7-Carboxy-CBD errechnet. Im zweiten Studienteil lag die mittlere maximale Serumkonzentration bei 38,4 ng/mL. Die terminale Halbwertszeit betrug 161,3 Stunden im Serum und die Rss lag bei 4,5.

Im ersten Studienteil ergab die Analyse der Verhaltensparameter, der Herzfrequenz und der Herzfrequenzvariabilität keine konsistent signifikanten Unterschiede zwischen der Behandlungs- und der Kontrollgruppe. Während des zweiten Studienteils unterschieden sich die täglichen Verhaltensbeobachtungen und die Cortisolwerte zwischen Behandlungs- und Kontrollgruppe ebenfalls nicht signifikant. Während des *Novel Object Test* und des Anhängertests wurden bei der Analyse der Reaktionen, Bewegungsmuster, HR, HRV und Cortisolspiegeln keine nachhaltig signifikanten Unterschiede zwischen den Gruppen festgestellt.

In dieser Studie wurden zum ersten Mal pharmakokinetische Parameter in Kombination mit dem Effekt von CBD auf Verhalten und Stress nach regelmäßiger oraler CBD-Verabreichung über zwei Wochen bei gesunden Pferden untersucht. Die pharmakokinetische Analyse zeigte einen umfassenden Metabolismus von CBD und eine hohe Verteilung in periphere Gewebe mit einer langen Eliminationsphase. Die Ergebnisse der

Verhaltensbeurteilungen lieferte keine gesicherten Hinweise auf einen stressmindernden oder sedierenden Effekt nach regelmäßiger Verabreichung von CBD in einer Dosierung von 3 mg/kg zweimal täglich. Die größte Limitation dieser Studie ist die geringe Stichprobengröße. Weitere Untersuchungen der potenziell stresslindernden Wirkung von CBD in Verbindung mit pharmakokinetischen Parametern sind erforderlich, um relevante CBD-Konzentrationen für Dopingkontrollen bei Pferdesportveranstaltungen bestimmen zu können. Zukünftige Studien sollten die Verabreichung höherer CBD-Dosen, beispielsweise 10 mg CBD/kg, in Erwägung ziehen und sich gezielt auf Pferde konzentrieren, die bekanntermaßen schnell Anzeichen von Nervosität und Stress zeigen.

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APPENDIX – SUPPLEMENTARY MATERIAL FOR PUBLICATION I

Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses

URL: https://www.doi.org/10.3389/fvets.2023.1234551#supplementary-material

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Supplementary Material

1 Supplementary Data

1.1 Preparation of urine samples for cannabinoid analysis

Cannabinoids were extracted from 5 mL urine by liquid/liquid extraction after addition of 50 µL of the internal standard mix (D3-CBD, D3-THC, D3-OH-THC, D3-COOH-THC, each at 1 µg/mL in methanol) and after hydrolysis of the phase-II metabolites. For the hydrolysis, samples were adjusted to pH 7 with 1 mL of 0.8 M phosphate buffer and then incubated after addition of 50 µL β-glucuronidase from *E. coli* at 50 °C for 1 h. Six mL of n-pentane were added and the mixture was shaken for 20 min and subsequently centrifuged at 600 g for 5 min. The n-pentane layer was separated and evaporated to dryness under reduced pressure. The dry residue was derivatized with 80 µL MSTFA/NH4I/ethanethiol 1000:2:3 (v:w:v) for 30 min at 80 °C and 6 µL were injected onto the gas chromatograph/mass spectrometry instrument.

1.2 Preparation of serum samples for cannabinoid analysis

Cannabinoids were extracted from 2 mL serum by liquid/liquid extraction after addition of 20 µL of the internal standard mix (D3-CBD, D3-THC, D3-OH-THC, D9-COOH-THC, each at 1 µg/mL in methanol). The mixture was adjusted to pH 5.2 with 100 µL of 4 M sodium acetate buffer. Five mL of a 50:50 (v:v) mixture of n-pentane and tert-butyl-methyl-ether were added and the mixture was shaken for 20 min and subsequently centrifuged at 600 g for 5 min. The organic layer was separated and evaporated to dryness under reduced pressure. The dry residue was derivatized with 80 µL MSTFA/NH4I/ethanethiol 1000:2:3 (v:w:v) for 30 min at 80 °C and 6 µL were injected onto the gas chromatograph/mass spectrometry instrument.

1.3 Gas chromatography/tandem mass spectrometry (GC/MS/MS)

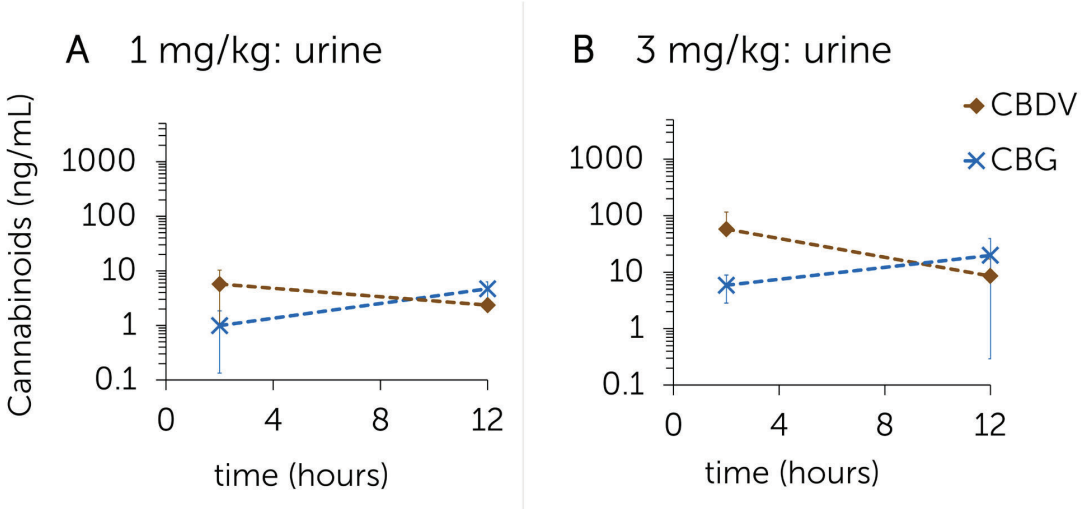
Analyses were performed using a Thermo Scientific TSQ 8000EVO tandem mass spectrometer coupled to a Thermo Scientific Trace 1310 gas chromatograph. A J&W Ultra 1 column (length 17 m, I.D. 0.2 mm, film thickness 0.11 µm) was employed, and helium was used as carrier gas at a constant pressure of 17.6 psi. An aliquot of 6 µL of the sample extract was injected into the GC/MS/MS system, which was operated in split mode (1:10). The GC temperature was ramped as follows: initial temperature = 157 °C, program rate = 20 °C/min to 325 °C, constant temperature = 325 °C for 1 min. The injection port and transfer line were heated to 300 °C. The trimethylsilylated analytes were measured using selected reaction monitoring (SRM) after electron ionisation (EI) and collision induced dissociation (CID) with argon as collision gas. The diagnostic ion transitions (listed as m/z), retention times (RT) and collision energies (CE) for each compound are presented in Table 1S.

Table 1S: Diagnostic ion transitions (specified as m/z), retention times (RT in min) and collision energies (CE in V) for each analyte and the corresponding internal standards (ISTD).

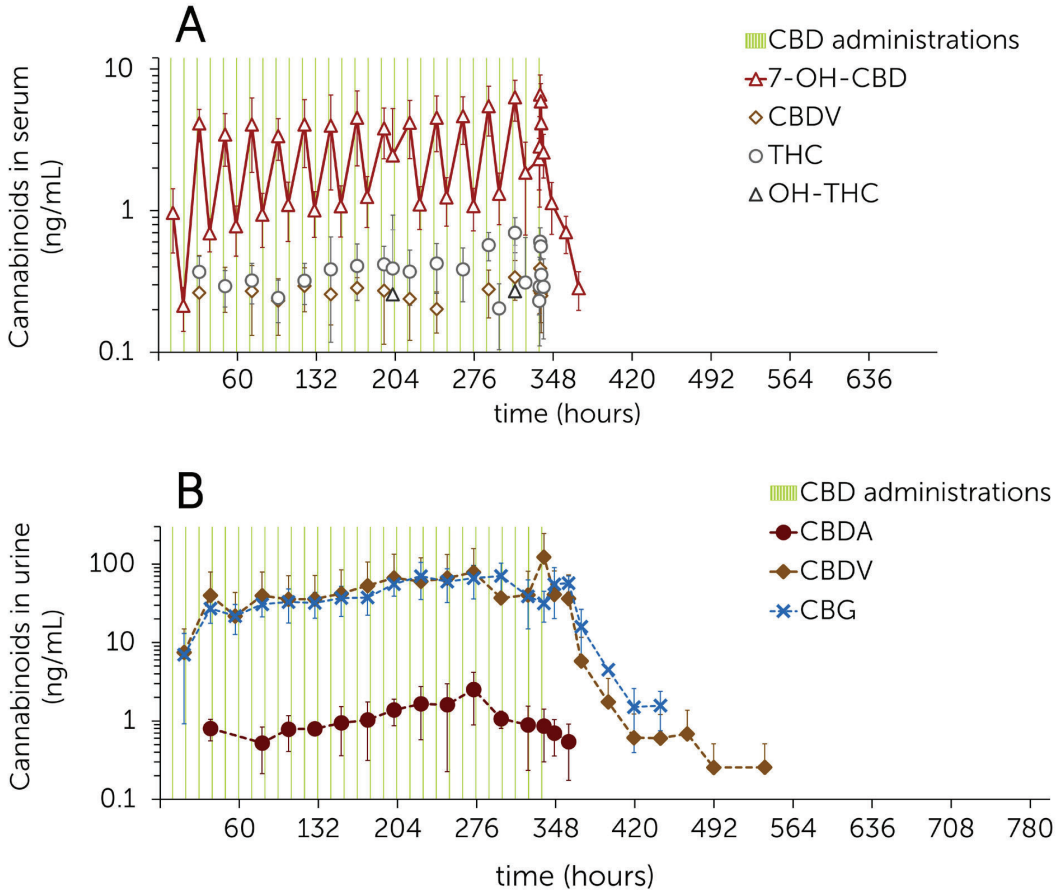
Cannabinoid	**RT**	**m/z**	**(CE)**	**ISTD**	**RT**	**m/z (CE)**
CBD	4.33	390/301	(8)	D3-CBD	4.32	393/304 (10)
CBDA	5.45	491/133	(29)	D9-Carboxy-THC	6.28	380/314 (10)
CBDV	3.63	362/273	(7)	D3-Hydroxy-THC	5.78	374/292 (13)
CBG	5.03	337/321	(9)	D3-CBD	4.32	393/304 (10)
7-COOH-CBD	5.70	443/119	(14)	D9-Carboxy-THC	6.28	380/314 (10)
7-OH-CBD	5.36	443/337	(9)	D3-Hydroxy-THC	5.78	374/292 (13)
COOH-THC	6.30	371/305	(12)	D9-Carboxy-THC	6.28	380/314 (10)
OH-THC	5.79	371/305	(7)	D3-Hydroxy-THC	5.78	374/292 (13)
THC	4.72	389/371	(11)	D3-THC	4.71	389/374 (10)

Abbreviations: CBD, cannabidiol; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBG, cannabigerol; 7-COOH-CBD, 7-carboxy-cannabidiol; 7-OH-CBD, 7-hydroxy-cannabidiol; THC, Δ9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; OH-THC, 11-hydroxy-Δ9-tetrahydrocannabinol.

2 Supplementary Figures



Supplementary Figure 1S: Mean ± standard deviation of cannabidivarin (CBD) and cannabigerol (CBG) in urine after single oral administration of CBD paste in two different doses (1mg/kg po (A); 3mg/kg po (B)).



Supplementary Figure 2S: Mean ± standard deviation of the following cannabinoid concentrations: 7-hydroxy-cannabidiol (7-OH-CBD), cannabidivarin (CBDV), Δ9-tetrahydrocannabinol (THC) and 11-hydroxy-THC (OH-THC) in serum (A), and cannabidiolic acid (CBDA), CBDV and cannabigerol (CBG) in urine (B) following multiple administrations of CBD paste (3 mg/kg po) twice daily over two weeks with subsequent sample collection.

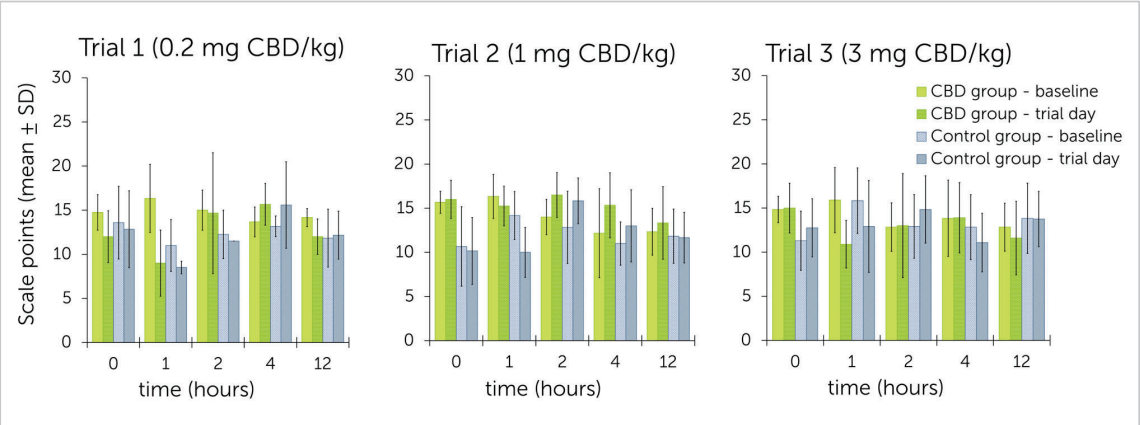
APPENDIX – SUPPLEMENTARY MATERIAL FOR PUBLICATION II

Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2)

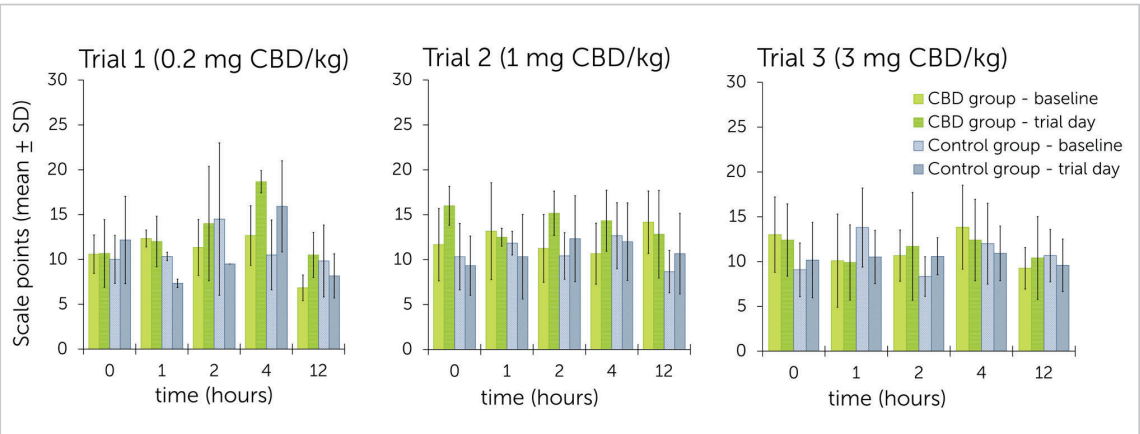
URL: https://www.doi.org/10.3389/fvets.2023.1305868#supplementary-material

SUPPLEMENTARY MATERIAL

1 Figures

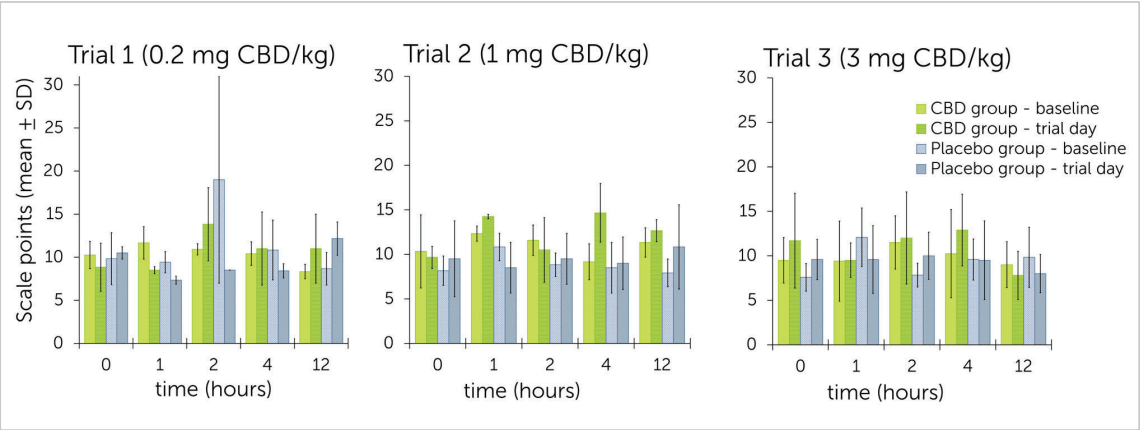


Supplementary Figure 1S: Sedation scores after acoustic stimulation with a clicker following single oral administration of cannabidiol (CBD) paste in escalating doses (0.2 mg CBD/kg; 1 mg CBD/kg; 3 mg CBD/kg) - comparison between values obtained on baseline and trial day for the treatment and control group. Higher scale points relate to a higher level of sedation (Table 1).



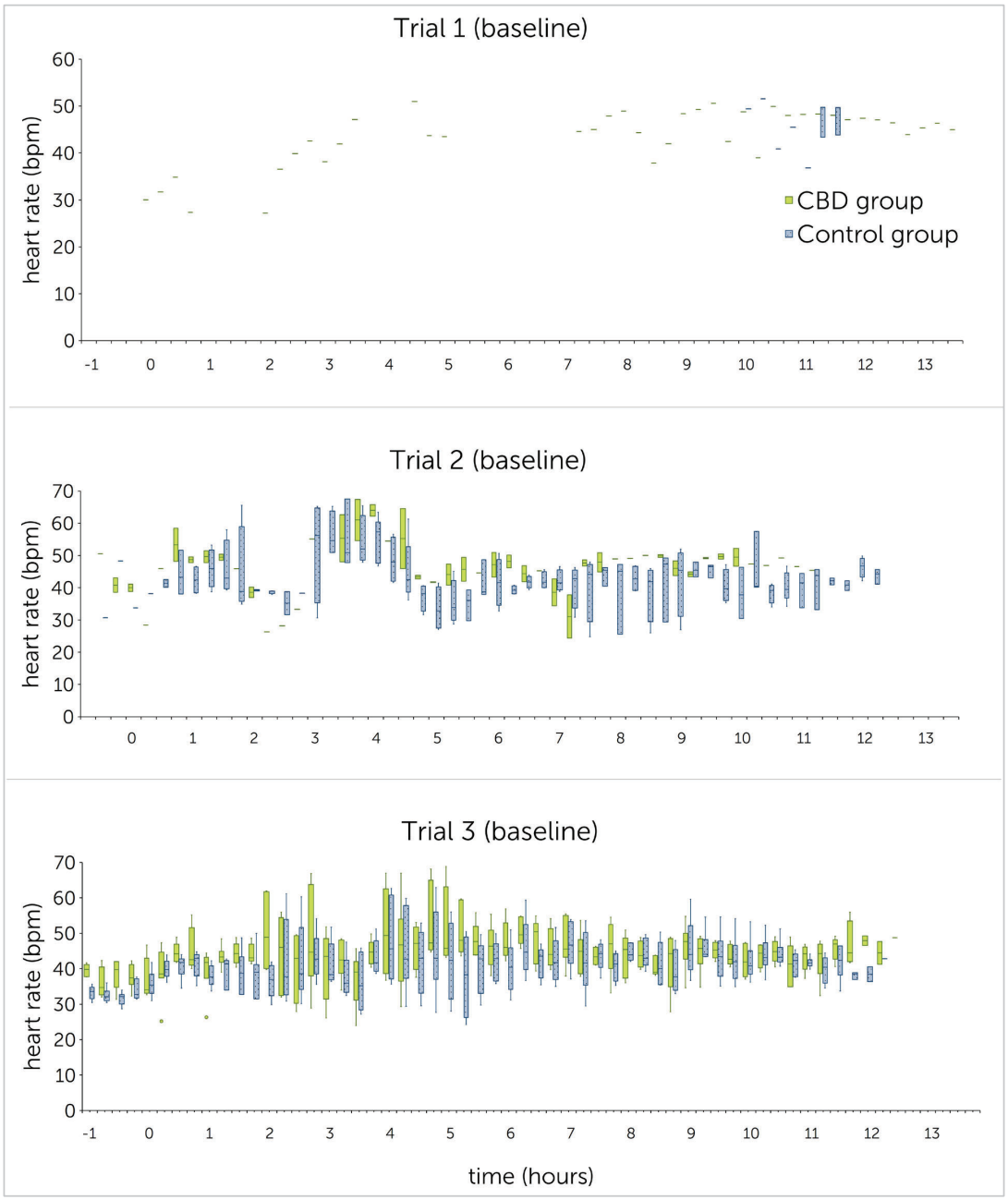
Supplementary Figure 2S: Sedation scores after acoustic stimulation with a plastic bag following single oral administration of cannabidiol (CBD) paste in escalating doses (0.2 mg CBD/kg; 1 mg CBD/kg; 3 mg CBD/kg) - comparison between values obtained on baseline and trial day for the treatment and control group. Higher scale points relate to a higher level of sedation (Table 1).

SUPPLEMENTARY MATERIAL



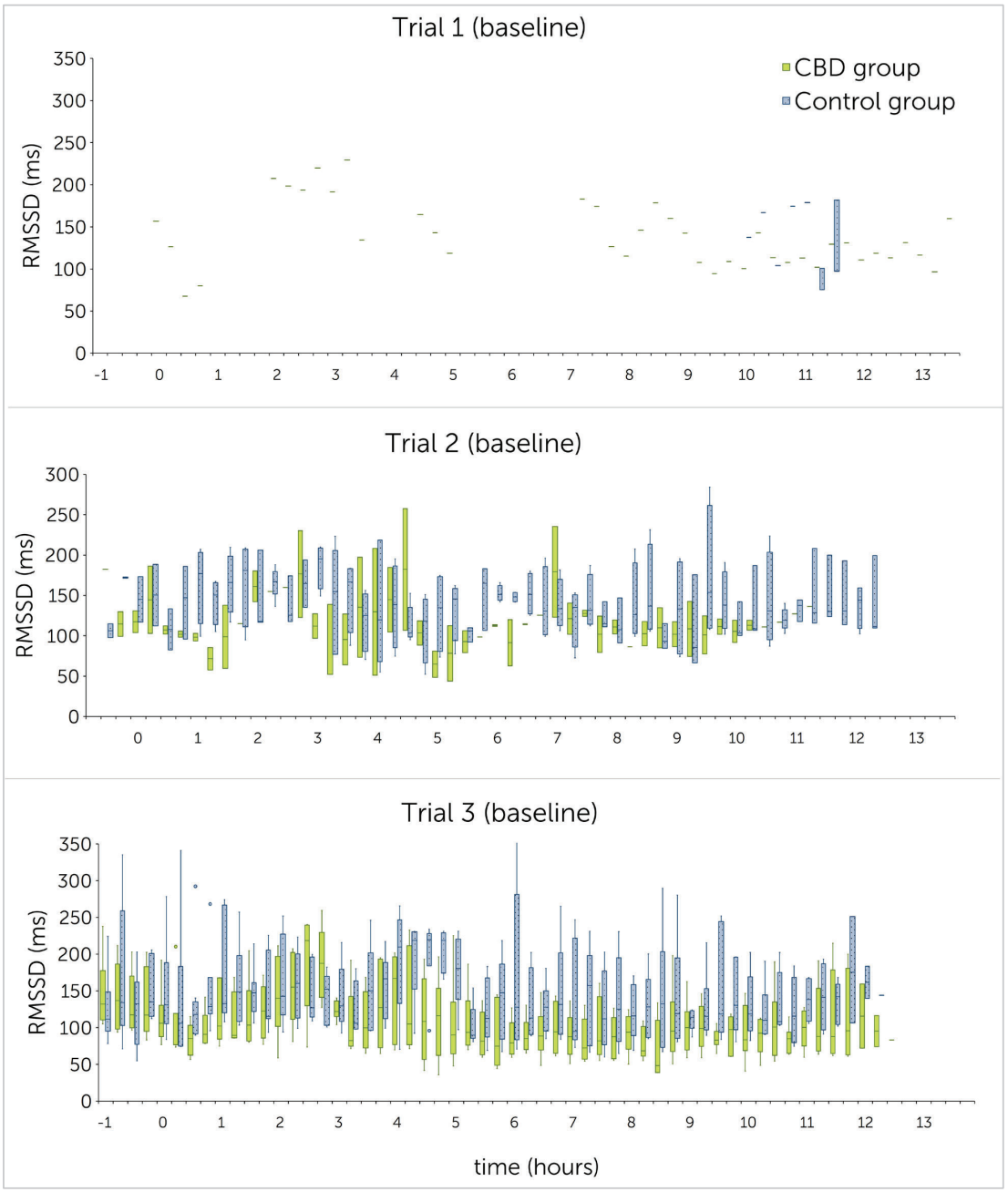
Supplementary Figure 3S: Sedation scores after visual stimulation with a pink cloth following single oral administration of cannabidiol (CBD) paste in escalating doses (0.2 mg CBD/kg; 1 mg CBD/kg; 3 mg CBD/kg) - comparison between values obtained on baseline and trial day for the treatment and control group. Higher scale points relate to a higher level of sedation (Table 1).

SUPPLEMENTARY MATERIAL



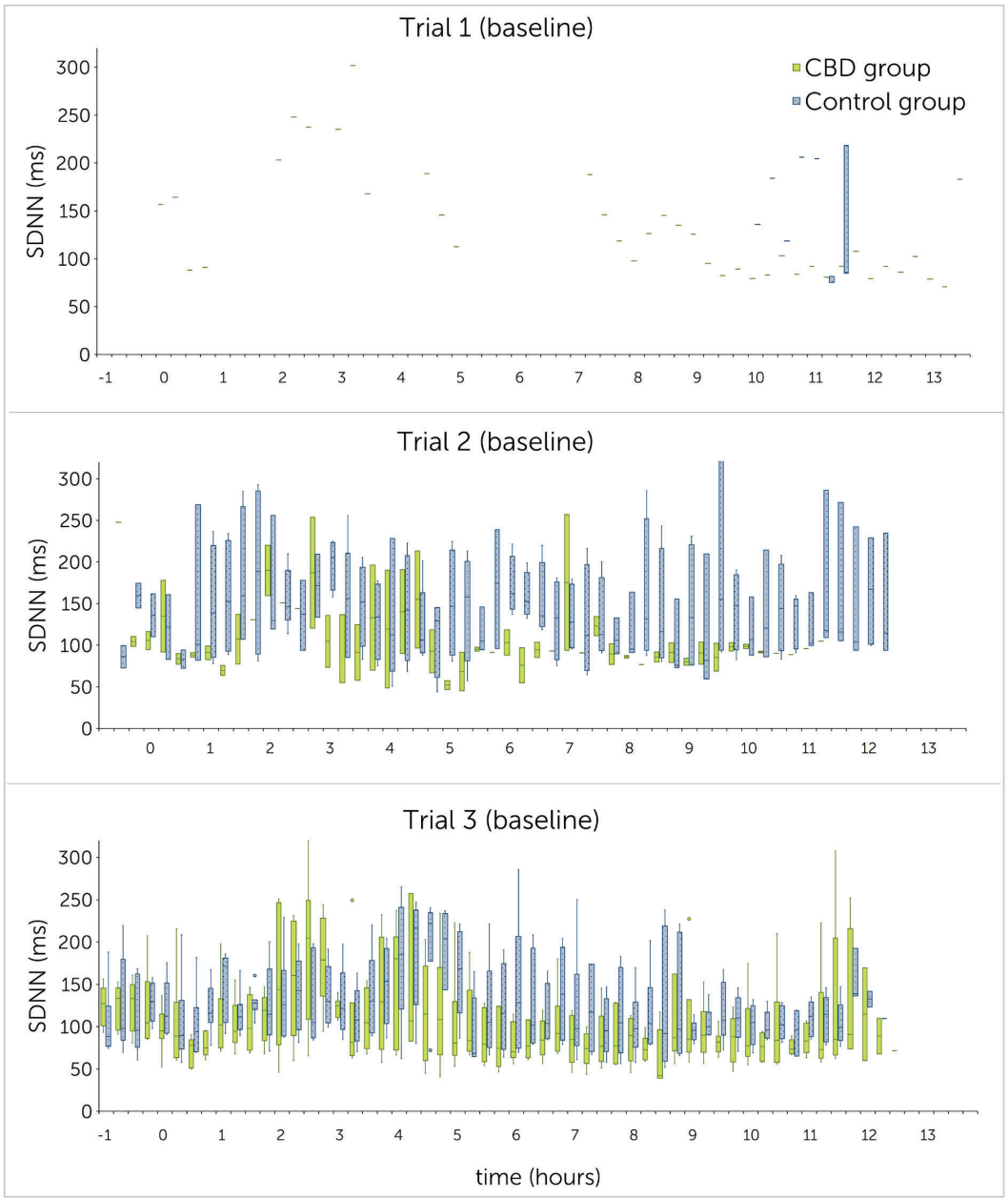
Supplementary Figure 4S: Heart rates (beats per minute (bpm)) prior to single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW), displayed in 15-minute sections over 12 hours. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

SUPPLEMENTARY MATERIAL



Supplementary Figure 5S: Root mean square of successive R-R interval differences (RMSSD) in milliseconds (ms) prior to single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW), displayed in 15-minute sections over 12 hours. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

SUPPLEMENTARY MATERIAL

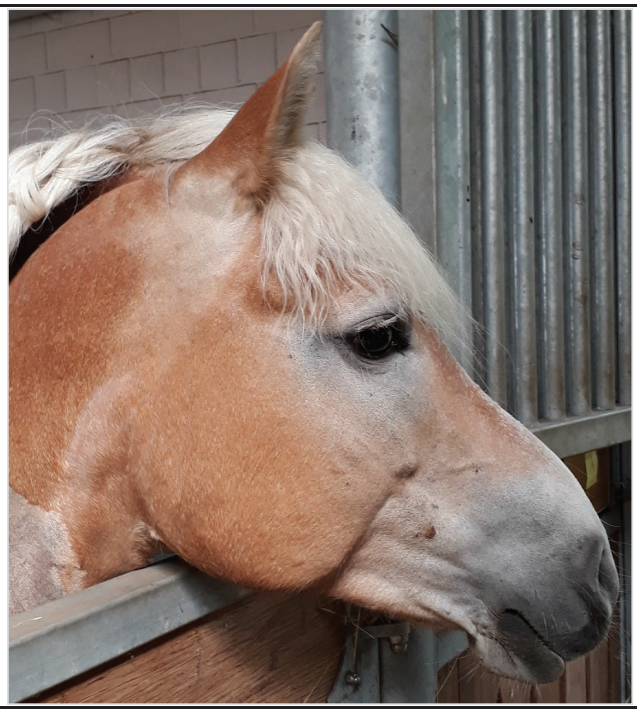


Supplementary Figure 6S: Standard deviations of R-R intervals (SDNN) in milliseconds (ms) prior to single oral administration of cannabidiol (CBD) in three escalating doses (0.2 mg CBD/kg BW; 1 mg CBD/kg BW; 3 mg CBD/kg BW), displayed in 15-minute sections over 12 hours. Due to technical issues, the trial 1 R-R-interval data are partly incomplete.

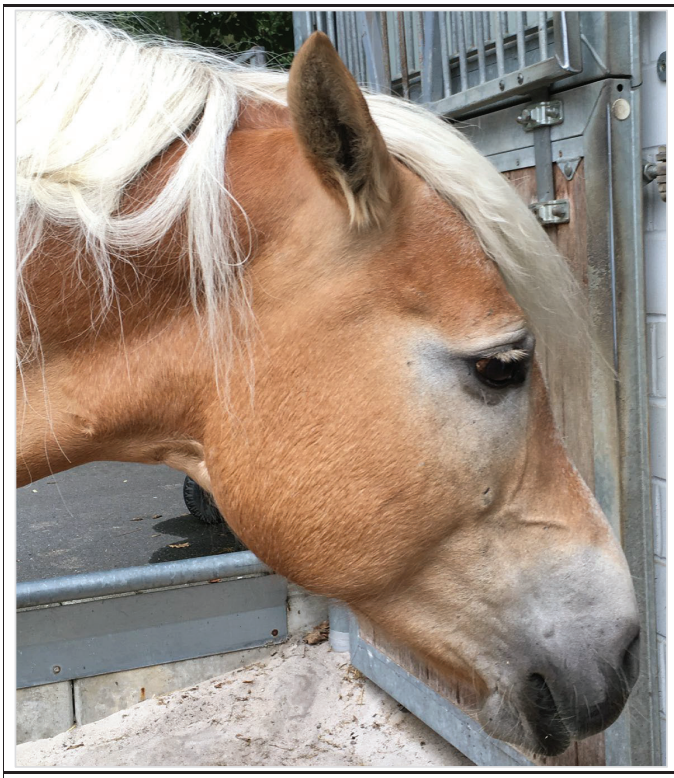
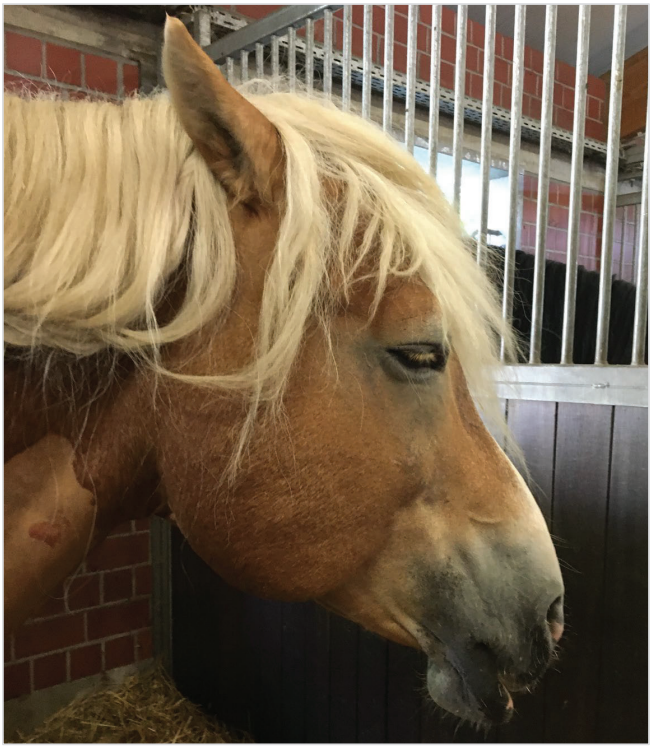
SUPPLEMENTARY MATERIAL

2 Tables

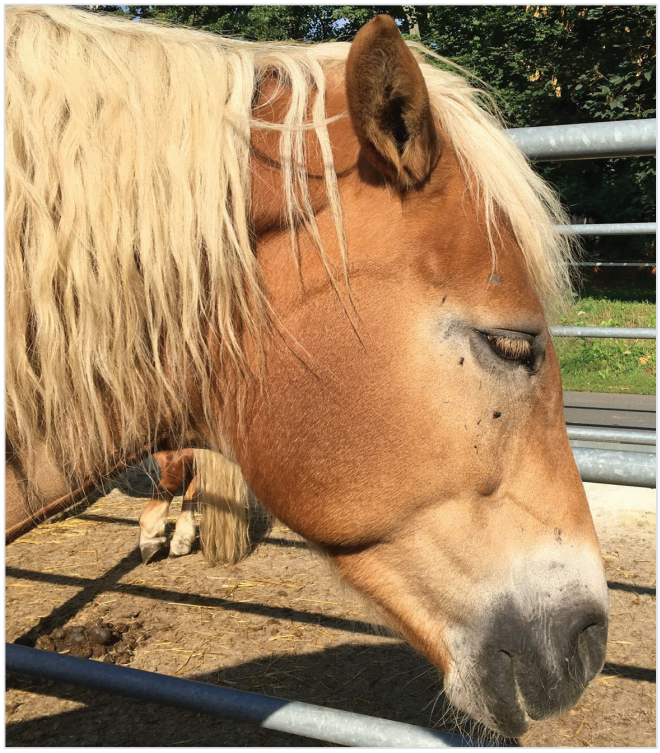
Supplementary Table 1S: Examples of the facial expression scale (Table 2).

	**Orbital opening** 2 Eyes completely open
	Position of ears 2 Pointed, position of attention
	Chewing muscles 2 Moderately present
	Lips 2 Loose touching of lips
	Nostrils 1 Dilated, outer ring clearly visible
	Total sum: 9
	**Orbital opening** 2 Eyes completely open
	Position of ears 2 Pointed, position of attention
	Chewing muscles 2 Moderately present
	Lips 2 Loose touching of lips
	Nostrils 2 Non-dilated nostrils
	Total sum: 10

SUPPLEMENTARY MATERIAL

	**Orbital opening** 3 Eyes partially open (> 50%) **Position of ears** 3 Asymmetrical; one ear hanging **Chewing muscles** 2 Moderately present **Lips** 2 Loose touching of lips **Nostrils** 3 Small nostrils, relaxed outer ring **Total sum: 13**
	**Orbital opening** 4 Eyes almost/completely closed (< 50%) **Position of ears** 4 Wide opening between ear tips **Chewing muscles** 2 Moderately present **Lips** 4 Pronounced relaxation/hanging of one lip **Nostrils** 2 Non-dilated nostrils **Total sum: 16**

SUPPLEMENTARY MATERIAL

	**Orbital opening** 4 Eyes almost/completely closed (< 50%) **Position of ears** 4 Wide opening between ear tips **Chewing muscles** 3 Not present **Lips** 3 Slight relaxation of one lip **Nostrils** 3 Small nostrils, relaxed outer ring **Total sum: 17**

APPENDIX – SUPPLEMENTARY MATERIAL FOR PUBLICATION III

Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2)

URL: https://www.doi.org/10.3389/fvets.2023.1305873#supplementary-material

SUPPLEMENTARY MATERIAL

1 Assessment of cortisol levels

1.1 Preparation of serum and saliva samples for cortisol analysis

Aliquots of 0.5 mL serum or saliva were fortified with 25 ng/mL or 0.5 ng/mL of the internal standard D4-hydrocortisone, respectively. After pH adjustment to 9.6 with a 2:1 mixture of solid $NaHCO_3/K_2CO_3$, samples were extracted with 5 mL *tert*-butyl methyl ether (tBME) for 20 minutes on a horizontal shaker. Centrifugation for 5 min at 600 g enabled the separation of the ethereal layer, which was evaporated. The residue was reconstituted in 2 mL $MeOH/H_2O$ (95/5, v/v). The methanolic layer was washed with 5 mL *n*-pentane for 5 minutes and the supernatant was separated by centrifugation and discarded. The methanolic layer was evaporated and the residue reconstituted in 100 µL LC buffer consisting of ammonium acetate (5 M)/acetonitrile (3/2, v/v) and 1% acetic acid. Aliquots of 10 µL were injected into the LC-MS/MS instrument.

1.2 High performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) for detection of cortisol in serum and saliva samples

LC-MS/MS analyses were performed on an Agilent Series 1260 liquid chromatograph (Waldbronn, Germany) coupled to a 5500 QTrap triple-quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray ionisation (ESI) interface. The column was a Nucleodur C18-Pyramid-column with dimensions of 2 x 50 mm and particle size of 3 µm protected by a guard column from Macherey-Nagel (Düren, Germany). The LC conditions were as follows: mobile phase A = ammonium acetate buffer (5 mM, pH 5, containing 0.1% acetic acid), B = acetonitrile, flow rate 0.35 mL/min, gradient 0% B → 100% B in 7 minutes, re-equilibration time 4.5 minutes at 0% B. Samples were measured in the negative operation mode at an interface temperature of 450 °C with an ion spray voltage (ISV) of -4500 V. Diagnostic ions of the analytes were generated by collision induced dissociation (CID) with nitrogen at a collision gas pressure of 2.3 x 10-3 Pa. Multiple reaction monitoring (MRM) experiments were performed on the most abundant ion transitions, which were optimized by support of the software Analyst 1.6 after infusion of the corresponding reference solutions. Selected quantifier MRM transitions were m/z 421/282 and 425/335 for the hydrocortisone acetate adduct and the D4-hydrocortisone acetate adduct, respectively.

1.3 Method validation for the analysis of cortisol

Validation for the quantification of cortisol in plasma samples was conducted considering precision and accuracy, stability, lower limit of detection (LLOD), lower limit of quantification (LLOQ), linearity, selectivity and robustness. A separate validation for cortisol in saliva was not performed. Instead, a calibration line was individually prepared for each batch of post administration samples and used for calculation of the cortisol concentrations within this batch. Cortisol was identified by three specific ion transitions. Additionally, the presence of cortisol was confirmed by the product ion scan of the molecular ion ($M - H^+$) of its acetate adduct. Ten different serum samples showed no interfering signals at the retention time of (endogenous) cortisol that could interfere with the signal identification and peak integration of cortisol.

Precision, accuracy and stability were determined as described for the validation of cannabinoids (47). Table 1 summarizes the results with respect to the cortisol concentration levels selected for the

tested validation parameters. A signal-to noise ratio of 3 and 9 was used to determine LLOD and LLOQ in equine serum and saliva, respectively. A series of 6 determinations at the concentration of the LLOQ was used for verification. The linearity of cortisol in serum was examined by a series of 9 different concentrations spiked into a cortisol stripped serum (SeraSub^{TM}, CST Technologies, Great Neck, USA). Linearity of cortisol in saliva was derived from 8 calibrators with water as the surrogate matrix for saliva. A weighting factor of 1/x was selected for both calibration lines. Correlation factors (R^2) were > 0.98 for both calibration curves and measured concentrations remained within the acceptance range of 85 - 115% of the theoretical cortisol concentration. Robustness was determined at a concentration of 20 ng/mL following the validation design for cannabinoids (47). All ten serum samples showed signals for cortisol with reproducible ion ratios. Relative retention time shifts were within acceptable ranges below 0.8%.

Table 1: Validation results for cortisol.

Analyte	Matrix	LLOD [ng/mL]	LLOQ [ng/mL]	Intra-day Precision CV [%] at 1/20/100 ng/mL	Inter-day Precision CV [%] at 1/20/100 ng/mL	Accuracy RE [%] at 1/20/100 ng/mL	Stability [%] at 20 ng/mL
Cortisol	Serum	0.1	0.2	3.8/6.7/5.7	4.7/5.4/11.0	3.3/4.0/-6.5	96
	Saliva	0.02	0.05	n.a.	n.a.	n.a.	n.a.

Abbreviations: n.a.: not applicable.

LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

I. Eichler F, Poźniak B, Machnik M, Schenk I, Wingender A, Baudisch N, Thevis M, Bäumer W, Lischer C and Ehrle A (2023): Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses. Front Vet Sci: 10:1234551. https://doi.org/10.3389/fvets.2023.1234551

II. Eichler F, Ehrle A, Jensen KC, Baudisch N, Petersen H, Bäumer W, Lischer C and Wiegard M (2023): Behavioral observations, heart rate and heart rate variability in horses following oral administration of a cannabidiol containing paste in three escalating doses (part 1/2). Front Vet Sci: 10:1305868. https://doi.org/10.3389/fvets.2023.1305868

III. Eichler F, Ehrle A, Machnik M, Jensen KC, Wagner S, Baudisch N, Bolk J, Pötzsch M, Thevis M, Bäumer W, Lischer C and Wiegard M (2024): Behavioral observations, heart rate and cortisol monitoring in horses following multiple oral administrations of a cannabidiol containing paste (part 2/2). Front Vet Sci: 10:1305873. https://doi.org/10.3389/fvets.2023.1305873

Presentations at scientific conferences

Oral presentations

- European Association for Veterinary Pharmacology and Toxicology (EAVPT) Congress 2023: Pharmacokinetic modelling of orally administered cannabidiol and implications for medication control in horses (In: J Vet Pharmacol Ther; 46: 57–58. doi: 10.1111/jvp.13227.)
- 6. Internationaler Kongress zur Pferdemedizin/Tagung der DVG-Fachgruppe Pferdekrankheiten 2023: Orale Cannabidiol-Gabe: Dopingrelevanz und Einfluss auf das Pferdeverhalten (In: Tagungsband Pferdekrankheiten / Pferdeophthalmologie. Verlag der DVG Service GmbH, 1. Auflage Gießen 2023. ISBN 978-3-86345-699-3).
- Berliner Turniertierärztemeeting 2023: Cannabidiol bei Sportpferden: Wirkung und Doping.

Poster presentations

- 4th European College of Veterinary Sports Medicine and Rehabilitation (ECVSMR) Scientific Meeting, 2023: Cannabidiol in horses – influence on stress parameters and implications for medication control (In: Proceedings, ECVSMR 2023. ISSN: 2510-8093)

ACKNOWLEDGEMENTS

First and foremost, I thank Christoph Lischer and Anna Ehrle for trusting me this project. It was an absolute joy to work with you and benefitting from your experience. Thank you for your confidence in me to handle this study and grow with every step along the way. Your kindness and quick support in all matters small and big were the key to the successful completion of this project.

A major thank you to Wolfgang Bäumer for the continuous guidance in this project. Your commitment in establishing the connection with Wrocław and help in delving deep into the world of pharmacokinetics have significantly contributed to the success of this study. I greatly appreciate your support and trust, particularly in the presentation at the EAVPT Congress 2023.

Mechthild Wiegard, thank you for your incredible support spanning from the animal testing application to all matters cortisol and behaviour, and your flawless hand in sewing electrode belts. Our diligent planning sessions in your office were always accompanied by a joyful atmosphere.

Many thanks to Marc Machnik and Ina Schenk for accompanying the project right from the beginning and your invaluable advice on how to handle the many, many samples. I am also grateful to the whole team at DSHS Köln, especially Anke Wingender, Sabrina Wagner and Mario Thevis, for the exceptional analytical work.

A special thank you to Błażej Poźniak for the excellent support and forward thinking in developing the pharmacokinetic model. The visit to Wrocław proved invaluable, significantly enhancing this project and making a substantial contribution.

Conny Pint and Daniela Wimmer, this project would not have been possible without you. Thank you for contributing the CBD products, for the perfect preparation and blinding of the pastes, and for your trust in our study execution.

A huge thank you to all my helpers at the Equine Center Bad Saarow and diligent Equine Sciences students: Natalie, Julia, Magdalena, Hannah, Lena, Clara, Elisa, Franziska, Anja, Katharina and Jenny. Your excellent caretaking of the horses and extraordinary work ethic in analysing the behavioural data sets played a pivotal role in the success of this project. I wish I could have gifted you all your own favourite horse.

To the whole team at the Equine Center, especially Johannes Handler and Franziska Görwitz, thank you for the warm welcome at your stables and your friendly assistance in all practical matters.

Lastly, to the people in my life, my family and my friends. Thank you for your inspiration, your encouragement and your love.

FUNDING

The study was funded by the Freie Universität Berlin, the German Equestrian Federation (FN) and Herosan healthcare GmbH. Herosan healthcare GmbH was not involved in the study design, collection, analysis, interpretation of data or the writing of this thesis. The article processing charges for the publications were funded by the Open Access Publication Fund of Freie Universität Berlin.

CONFLICT OF INTEREST

The author of this thesis declares no conflict of interest.

DECLARATION OF ORIGINALITY

Hereby, I declare that the present thesis was prepared by myself. I assure that I exclusively used the mentioned sources and facilities.

Berlin, 01.10.2024

Fabienne Eichler