Residue profiles and therapeutic applications of unapproved anti-inflammatory drugs in cattle

by

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B.S., Kansas State University, 2022
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Disease and routine management procedures result in cattle regularly experiencing pain, inflammation, or stress. The 'Five Domains' animal welfare paradigm establishes that animals should have freedom from pain, injury, and disease and freedom to express normal behaviors. Effectively managing pain and inflammation due to disease and management procedures can meet these basic welfare needs. Non-steroidal anti-inflammatory drugs and glucocorticoids are commonly used to treat pain, inflammation, and stress. Because there are few approved anti-inflammatory drug options for cattle in the United States, veterinarians must use drugs in an extra-label manner in certain situations. However, for extra-label drug use (ELDU) to be legal, veterinarians must determine a conservative withdrawal interval (WDI) to avoid violative residues in edible tissues and ensure the treated animal does not enter the food chain prior to the end of that period. Variability in animal populations – whether due to age, breed, physiologic status, or disease state—can alter the pharmacokinetics of drugs. Thus, it is prudent to generate data to enable estimation of WDI for drugs in healthy populations and those of interest for a particular drug regimen. Industrial hemp (IH) is being evaluated in cattle as a novel, sustainable feed source and for possible therapeutic applications. Inclusion of IH in cattle feed or as a drug is currently illegal, in part due to concerns over the transfer of cannabinoids into edible tissues. However, data supporting tissue cannabinoid concentrations and therapeutic effects of IH are of interest to both promote and inform its approval as a feed ingredient.

This dissertation begins with a review of unapproved anti-inflammatory drugs pharmacokinetics and current IH research in cattle. Further chapters discuss residue depletion profiles for salicylic acid in milk following aspirin treatment in cows and for cannabinoids in tissues following IH administration in steers. Additional pharmacodynamic and pharmacokinetic

considerations for IH in cattle are explored through (1) evaluation of the effects of IH and repeated transportation events on measures of stress and inflammation and (2) description of the plasma cannabinoid profile after long-term IH administration.

Salicylic acid was detected in the milk and WDI were estimated for treated cows. Results indicated that a 120 h to 156 h WDI may be appropriate; this is longer than the previous 24-hour recommendation. Following aspirin administration, prostaglandin production was reduced for up to 12 h. When this aspirin report was initially published, extra-label aspirin use was common in dairy cattle and the FDA used enforcement discretion in not pursuing action against administration of unapproved, over-the-counter marketed products. However, concerns with mass aspirin treatment of dairy cows during the highly pathogenic avian influenza outbreak have since prompted prohibition of extra-label aspirin use.

Following administration of IH inflorescence to Holstein steers, a variety of cannabinoids, including the psychoactive $\Delta 9$-tetrahydrocannabinol (9-THC) and the bioactive cannabidiol (CBD), were detected in tissues. Both CBD and 9-THC accumulated in adipose tissue. Slow depletion of some cannabinoids resulted in WDI estimates with a large degree of extrapolation. Exposure estimates for human consumers demonstrated that a single demographic (newborns) exceeded the most conservative international toxicity threshold for 9-THC. Additional research should establish safe thresholds of cannabinoids in younger (vulnerable) demographics.

Results showed that IH administration, in a complex relationship with transportation and time, may reduce prostaglandin E_2 metabolites (a measure of inflammation). Transported cattle had increased activity during transportation. However, both transported and non-transported cattle lay down more following the return of the transported group. Transport elicited changes in blood

parameters and cortisol that are consistent with previous reports. These data will enable the design of impactful future studies regarding IH use in cattle.

During long-term IH administration, predominantly acidic cannabinoids were detected in plasma. (−)-7-nor-7-carboxy cannabidiol (CBD-7-acid) reached the highest concentrations and depleted the most slowly after cessation of IH administration. A handful of cannabinoids, including CBD-7-acid, were detected in cattle not receiving IH. This suggests that cattle not receiving IH could have detectable cannabinoid concentrations in the blood if cohoused with animals that are exposed to IH.

In conclusion, our results provided information for generation of WDI following administration of aspirin to lactating cows. Our data provide information on target tissues and marker residues for post-slaughter surveillance and a suggest a promising candidate for ante-mortem testing of IH exposure. Treatment with IH may reduce some inflammatory markers. Further work should continue to evaluate the food safety profile of cattle exposed to IH as well as the potential therapeutic benefits of IH administration.

Key words: anti-inflammatory, aspirin, industrial hemp, residue, transportation, withdrawal interval

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Acknowledgements

Dedication

Raising a graduate student takes a village. This dissertation is dedicated to the many people in my village, particularly my family and the mentors who encouraged me towards research and veterinary medicine.

I especially dedicate this to my late grandparents, Conrad and Terri Pyle and Lois Meeth—who went above and beyond to support my education—and to Dr. Warren Beard, who inspired me on the path to become a veterinarian and whose quiet encouragement, support, and praise meant the world to me.

Chapter 1 - Literature review: Residue pharmacology of extra-label anti-inflammatory agents in cattle

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ABSTRACT

Cattle regularly experience pain and inflammation due to disease or routine management procedures. Appropriate analgesic and anti-inflammatory therapy, such as with non-steroidal anti-inflammatory drugs or glucocorticoids, is critical to protect animal well-being. Industrial hemp is being evaluated in cattle for its potential as a novel, sustainable feed source and for possible therapeutic applications. There are limited approved anti-inflammatory drug options for cattle in the United States, necessitating extra-label drug use (ELDU) in some scenarios. One requirement in order for ELDU to be legal is for veterinarians to establish and enforce a conservative withdrawal interval to avoid violative residues in edible tissues. In the case of additional questions or concerns regarding specific instances of ELDU, readers are encouraged to contact the Food Animal Residue Avoidance Databank. The objective of this review is to summarize the available literature on tissue residue profiles and relevant pharmacokinetic parameters of unapproved anti-inflammatory drugs or ELDU of anti-inflammatory compounds in cattle.

Key words: cattle, extra-label, industrial hemp, NSAID, residue, steroids

INTRODUCTION

Cattle experience pain and inflammation secondary to a variety of physiologic processes, routine management procedures, and disease states (Kleinhenz et al., 2021). Mitigating pain and inflammation is necessary to improve cattle welfare and often improves production performance. Non-steroidal anti-inflammatory drugs (NSAID) and glucocorticoids, or steroids, are the most commonly used drug classes when treating inflammatory or painful states. While NSAID work by inhibiting cyclooxygenase enzymes to prevent cleavage of arachidonic acid into prostanoids, steroids work farther upstream, preventing phospholipase A_2 and subsequent release of

arachidonic acid from cell membranes (Lees, 2018). There is currently one non-steroidal anti-inflammatory drug (NSAID)—flunixin— approved in the United States for control of pain and inflammation in cattle and two steroids, dexamethasone and isoflupredone, approved for treatment of bovine primary ketosis and inflammatory conditions. Veterinarians and producers are thus relatively limited in their choices of approved drugs to manage pain and inflammation in cattle.

Over the past decade, there has been growing interest in industrial hemp (IH) in the United States, as two consecutive Farm Bills reclassified it as a non-controlled substance and allowed both research and commercial cultivation (Johnson, 2021). The primary market in the United States is for cannabidiol (CBD) oil, but extraction of this product generates plant material viewed as waste (Altman et al., 2024, Irawan et al., 2025a). Because cattle and other ruminants can convert fibrous plant material into edible tissues (i.e. meat and milk), there is an opportunity to use IH and its byproducts as livestock feed. This would not only improve the sustainability of IH production, but IH also offers a favorable nutrient profile and may have benefits on cattle health and performance (Kleinhenz et al., 2020b, Altman et al., 2024, Irawan et al., 2025a). Certain bioactive compounds, such as cannabinoids, within IH may also have therapeutic applications in cattle. Various cannabinoids have been shown to have anti-inflammatory, antioxidant, and even anticancer activities in in vitro, ex vivo, and some in vivo models (Malfait et al., 2000, Costa et al., 2004, Takeda et al., 2008, Ruhaak et al., 2011, Pellati et al., 2018, Shin et al., 2024). Our group has previously shown that administration of multiple doses of unextracted IH inflorescence reduces cortisol and prostaglandin E_2 concentrations in Holstein steers (Kleinhenz et al., 2022). Despite these potential benefits, the presence of cannabinoids, including the psychoactive compound, $\Delta 9$-tetrahydrocannabinol (9-THC) and bioactive

cannabinoid, CBD, raises concern for consumer safety if exposed to tissues from animals fed IH products. Pending additional research and regulatory action regarding the safety of IH products in livestock, it may represent a novel anti-inflammatory therapy for cattle.

While there is a strong market for approval of pharmaceuticals for humans suffering from painful or inflammatory conditions, the prolonged and expensive drug approval process is often prohibitive for food animal drug development. However, veterinarians have an obligation to relieve animals' suffering and protect animal welfare, and producers are driven to address pain in their cattle to improve animal well-being and increase performance. The American Veterinary Medical Association and the American Association of Bovine Practitioners have published position statements and guidelines regarding pain mitigation during management procedures. Depending on the characteristics of the individual animal (i.e. signalment, comorbidities), disease state or management procedure, and class of cattle involved, approved anti-inflammatory drugs may not always be an appropriate choice. Use of unapproved NSAID or use of approved anti-inflammatory drugs in an off-label manner is common in the cattle industry, both due to the necessity of treating painful conditions and due to unscrupulous practices.

The Animal Medicine Drug Use and Clarification Act (AMDUCA) of 1994 legalized extra-label drug use (ELDU) in veterinary species, but requires veterinarians to adhere to specific guidelines (FDA, 1996). In order to use a drug in an extra-label manner, a valid veterinary-client-patient relationship must be established, the veterinarian may only use drugs approved by the Food and Drug Administration (FDA) for animals or humans, there must be a threat to the health or well-being of the animal (no ELDU to enhance production) if treatment is withheld, no drug may be used extra-label in the feed, and violative drug residues in edible tissues must be prevented by establishment and enforcement of a conservative drug withdrawal interval (WDI).

In addition, ELDU is only allowed when an approved drug is unavailable for the intended purpose, or the approved drug would be ineffective or in an inappropriate form.

The Food Animal Residue Avoidance Databank (FARAD) is a USDA-funded program that serves to protect the safety and quality of United States livestock markets by providing evidence-based information regarding WDI. The terms withdrawal time (WDT) and WDI both refer to the time from drug administration until depletion of drug below the established tolerance level. However, FARAD uses the term WDI to refer to estimates generated for ELDU, whereas WDT refers to the interval established for labeled use of an approved drug. Members of FARAD use a variety of tools to generate WDI based on available scientific literature. Establishment of a WDT requires a rigorous set of animal studies to determine marker residues and depletion times (FDA CVM, 2015, 2022). Various approaches, such as linear regression, physiologically based pharmacokinetic modeling, and population pharmacokinetics allow for WDI estimation using existing data.

Knowledge of ELDU and its effects on the disposition of drugs in cattle is critical for avoiding violative residues, per AMDUCA guidelines. Many reports have noted that using drugs in an extra-label manner can prolong the time required for residues to reach safe concentrations (Gehring et al., 2006, Deyrup et al., 2012, Smith, 2013, Mzyk et al., 2017, Sidhu et al., 2017, Greene and Martinez, 2023, Kleinhenz and Gorden, 2025). Because WDT are usually generated based on data acquired from healthy animals, even using drugs per label directions requires discretion if being administered in certain disease states.

The objective of this review is to summarize the available literature regarding the tissue pharmacokinetics and depletion of unapproved anti-inflammatory drugs and ELDU of anti-

inflammatory drugs in cattle. This review will focus on non-steroidal anti-inflammatory drugs, steroids, and IH products.

METHODS

The literature search was broken into four categories: 1) cattle, 2) anti-inflammatory drugs, 3) unapproved status, 4) residue pharmacology. Search terms were aligned with MeSH results in PubMed; where specific phrases were of interest, these were included in quotation marks. After all categories were built individually, combined searches were performed in PubMed, Web of Science, Agricola, and CABI Digital Library. Due to accidental exclusion of a few drug terms and some anticipated results not being returned in the first search, four additional searches were constructed. Additional references not returned by the search were acquired from the FARAD database. Search terms and the number of results returned from each search can be found in the supplementary file.

All authors participated in search construction; one author (B.R.F.) was responsible for screening search results. Articles in a non-English language, those without an associated full text, and those that did not refer to cattle and a term from at least one other category were excluded during title screening. A total of 1,624 titles were advanced to the abstract screening phase. In that phase, articles were eliminated if they did not mention an aspect of live animal evaluation or pharmacokinetics. Articles that mentioned plasma but not tissues were still included and full texts reviewed as reference material but are not necessarily included in this report. A total of 174 articles were advanced to full-text review. Additional articles were eliminated during full-text review for lack of relevance, lack of *in vivo* data, including only surveillance-type data, or only including one timepoint (unless the available literature for that drug was deemed sparse). A literature review flowchart is shown in Fig. 1.1.

There were no relevant results returned for antihistamines or other natural anti-inflammatory drugs.

Non-steroidal anti-inflammatory drugs

Acetaminophen

Acetaminophen is not considered a typical NSAID but rather exerts its effects centrally on transient receptor potential vanilloid 1 and cannabinoid receptor 1 proteins (Ohashi and Kohno, 2020). There is no veterinary approval for acetaminophen in the United States, but there are approved human products available.

Acetaminophen has been investigated for use as an indirect marker of abomasal emptying rate, with a reported plasma half-life of 300 minutes following administration of 50 mg/kg acetaminophen in milk replacer (Marshall et al., 2005).

There have been reports of acetaminophen residues in urine of veal calves without history of drug exposure (Groot et al., 2023). Groot et al. (2023) investigated potential sources of residues and found that administration of a maximum allowable level of roughage in veal calf diets did not result in acetaminophen or metabolite concentrations above the limit of quantification in urine. The authors reported that veal calves treated with acetaminophen at 30 mg/kg in milk replacer 5 days a week on alternating weeks had concentrations up to 13,000 µg/kg in muscle, 43,000 µg/kg in liver, and 72,000 µg/kg in kidney (Groot et al., 2023).

Lautz et al. (2024) developed a PBPK model to predict tissue concentrations of various NSAID. They reported an *in vivo* liver clearance of 0.31 L/h/kg, derived from the literature. They reported the residue ratios of 1.14 for meat to kidney and 10.3 for meat to plasma.

There are no reports on milk residue profiles of acetaminophen. Considering that acetaminophen is a weak acid like other NSAIDs, there is potential for transfer to milk products.

There are reports of prolonged depletion of other drug residues in postpartum animals or those suffering from mastitis or other inflammatory processes, which could feasibly occur for acetaminophen as well (Lohuis et al., 1991, Kissell et al., 2015, Gorden et al., 2018a, Mzyk et al., 2018, Warner et al., 2020, Fritz et al., 2022, Mzyk et al., 2023, Kleinhenz and Gorden, 2025).

Aspirin

Aspirin is a member of the salicylate subgroup of the carboxylic acid class of NSAID (Lees, 2018). There is no approved veterinary product in the United States and only one approved prescription product for humans. Aspirin has historically been frequently used in dairy cattle and is one of two NSAID permissible in organic production (Smith et al., 2008, Smith, 2013, Fritz et al., 2022). While there are no approved veterinary products, numerous over-the-counter formulations are readily available in practical dosages for cattle. Due to its rapid half-life, relatively low use, and likelihood of an adverse human event, the FDA previously had viewed aspirin as a drug of low regulatory concern and had not taken action against its use, give its ability to enact enforcement discretion (Smith et al., 2008). However, in light of a drastic increase in usage in the dairy industry secondary to outbreaks of highly pathogenic avian influenza, the FDA released a letter reiterating the lack of any veterinary approval for aspirin (FDA, 2024). Although the approved human product, Vazalore, could theoretically be used in accordance with AMDUCA guidelines, administering an appropriate dose for cattle would be impractical. Any use of the unapproved, over-the-counter options marketed for veterinary species is illegal, as this represents extra-label use of an unapproved drug, which is not permitted under AMDUCA. Retailers should no longer be able to sell over-the-counter aspirin boluses. However, in the case of unauthorized access or accidental exposure, a brief discussion of the milk residue profile of aspirin will be summarized.

In a study evaluating the pharmacokinetics and milk residue profile of aspirin in postpartum dairy cattle, Fritz et al. (2022) estimated a 120 h WDI following exposure (200 mg/kg by mouth every 24 h for two doses). In the case that more than a third of bulk tank milk was comprised of milk from aspirin-treated cattle, the authors estimated a 156 h WDI.

Carprofen

Carprofen is a carboxylic acid of the 2-arylpropionic acid class (as are ibuprofen and ketoprofen) and is approved for veterinary use in the United States for dogs; there is an approval for cattle in the European Union (EU) (Lees, 2018). The injectable formulation of carprofen is a racemic mixture of the *R*-(−) and *S*-(+) enantiomers. The plasma pharmacokinetics in calves appear to be enantioselective, with the *R*-(−) enantiomer predominating (Lees et al., 1996, Brentnall et al., 2013). The plasma elimination half-life for the *R*-(−) enantiomer is slower and has been reported to be between 13 and 47 h when carprofen is administered as the sole agent, with most reports being in the range of 30 to 40 h (Lees et al., 1996, Brentnall et al., 2013). One study evaluating carprofen administered intravenously for five days reported terminal plasma elimination half-lives of 44.5 to 64.6 h in lactating cows; these cows were noted to be in the postpartum period, which could explain the higher values compared to the previously cited reports (Ludwig et al., 1989). The plasma elimination half-life of total carprofen in mastitic cows has been reported to be longer than healthy cows in an induced-mastitis model (Lohuis et al., 1991).

Carprofen residues have been reported to be in the highest concentrations in kidney and injection site when given intramuscularly (7.7 mg/g and 289 mg/g, respectively, with muscle, liver, and fat having lesser concentrations (Naidoo et al., 2018).

Carprofen generally appears to transfer minimally into milk. In a crossover study evaluating the comparative pharmacokinetics during induced mastitis, all milk samples were below 0.022 µg/kg (the limit of detection; LOD) in healthy cows and concentrations up to 0.164 µg/kg were reached in mastitic cows during the first 12 h after treatment with intravenous carprofen at 0.7 mg/kg (Lohuis et al., 1991). In a report of 6 postpartum cows administered 0.7 mg/kg carprofen intravenously once or daily for 5 days, most cows had milk concentrations below the LOD (25 ng/mL), with a few cows sporadically have samples with concentrations around 30 ng/mL after the fourth or fifth carprofen injections (Ludwig et al., 1989). Two of the cows in that report developed mastitis during the study; in those animals, milk concentrations were much higher (up to 218 ng/mL), even in healthy quarters. Once the mastitis was treated with antimicrobials, milk carprofen concentrations became undetectable.

Diclofenac

Diclofenac is a carboxylic acid in the thiopheneacetic acid subgroup (Lees, 2018). The only approved formulation in the United States is a topical cream for horses. There are a variety of formulations approved for humans.

Diclofenac has a reported plasma half-life ranging from 12.2 to 30.5 h; administration of the prodrug aceclofenac resulted in an estimated diclofenac half-life of 6.1 h (Mestorino et al., 2007, Taggart et al., 2007a, b, Galligan et al., 2016). Galligan et al. (2016) showed that aceclofenac is metabolized to diclofenac in *Bos taurus*, with rapid detection of diclofenac after aceclofenac administration. Diclofenac concentrations were equal to or greater than aceclofenac from 2 h onwards.

Green et al. (2006) evaluated the tissue concentrations of diclofenac after intramuscular administration in *Bos indicus* and *Bos taurus* cattle and found that diclofenac was at the highest

concentrations in kidney and liver. The estimated elimination half-lives of diclofenac from that study were 48, 45, 68, and 35 h in liver, kidney, muscle, and fat, respectively. Mestorino et al. (2007) administered a combination oxytetracycline-diclofenac product intramuscularly and reported that diclofenac concentrations were highest in injection site. Diclofenac concentrations were the second highest in adipose at the first sampling time (7 d), whereas concentrations were the second highest in liver and second lowest in fat at 14 d. This suggests a more rapid depletion of diclofenac from adipose than liver. Diclofenac was detectable in all tissues (liver, kidney, muscle, injection site, fat) at 14 d but undetectable in tissues at 21 d after administration. Taggart et al. (2007a, b) reported quantifiable diclofenac concentrations in liver and kidney up to 71 h, in muscle up to 46 h, and in plasma up to 48 h (plasma was not sampled past 48 h).

Considering the risk of mortality in vultures exposed to carcasses containing diclofenac—as is discussed in a majority of the publications listed above—the authors strongly advise veterinarians to consider other drugs for cattle in scenarios in which ELDU is necessary.

Dipyrone

Dipyrone has a veterinary approval for horses in the United States (Zimeta). Although it is no longer on the FDA's prohibited drug list, it is restricted from use in dairy cattle under the Grade A Pasteurized Milk Ordinance (FARAD, 2021). In addition, per the equine label warnings and a statement from the FDA, this product is not to be used in food animals (FDA CVM, 2019). Thus, it will not be discussed here.

Firocoxib

Firocoxib is a cyclooxygenase-2 selective inhibitor approved for horses and dogs in the United States (Lees, 2018).

The plasma elimination half-life of firocoxib in preweaned calves following intravenous administration is 6.7 h and after oral administration is 18.8 h (Stock et al., 2014). The same study found the terminal plasma elimination half-life following intravenous administration to be 37.2 h and reported an oral bioavailability of 98.4%. The authors discussed relatively low clearance and large volume of distribution as the major contributors to the long half-life. Although firocoxib is highly protein bound, like other NSAID, its lipophilic nature results in a larger volume of distribution than is typical for NSAID (Stock et al., 2014).

No data regarding tissue distribution were available in the literature. The lack of tissue data severely restricts the ability to estimate a WDI for this drug. Per AMDUCA guidelines, if there is insufficient data to estimate a WDI, the drug should not be administered. Readers are encouraged to contact FARAD with questions regarding their current WDI recommendations for firocoxib use in cattle.

Flunixin

Flunixin is a carboxylic acid within the anthranilic acid subgroup (Lees, 2018). It is approved for pain control due to foot rot, control of pyrexia secondary to bovine respiratory disease, endotoxemia and acute bovine mastitis and for control of inflammation due to endotoxemia in cattle. The non-analgesic approvals are in place for both intravenous and transdermal formulations. Following intravenous administration, there is a meat WDT of 4 d and milk WDT of 36 h (Ngoh et al., 2003). It is not for use in dry dairy cows, veal calves, or bulls intended for breeding. While use in non-veal calves is not explicitly prohibited, it would constitute ELDU. Intramuscular use of the intravenous product is not allowed, per the drug label: flunixin accumulates in and is slowly eliminated from damaged or inflamed tissues, leading to known risks of violative residues. The transdermal product is labeled in beef cattle 2 mo and

older and dairy cattle. It is not for use in beef and dairy bulls intended for breeding over 1 yr. Due to the lack of a WDT in calves, the transdermal formulation may not be used in replacement dairy heifers over 20 mo, dry dairy cows, beef calves less than 2 mo, dairy calves, or veal calves. The transdermal formulation has a meat WDT of 8 d and milk WDT of 48 h. The FDA tolerance for flunixin in the target tissue (liver) is 125 ppb; the tolerance for 5-hydroxyflunixin (5-OH-flunixin; the marker residue) in milk is 2 ppb.

The plasma half-lives of flunixin are approximately 2.5 to 8 h for intravenous, 3 to 8 h for intramuscular, 5 to 6 h for subcutaneous, and 5 to 7 h for oral administration (Anderson et al., 1990, Landoni et al., 1995a, Odensvik, 1995, Rantala et al., 2002, Kissell et al., 2012, Glynn et al., 2013, Shelver et al., 2013, Mzyk et al., 2017, Sidhu et al., 2017, Gorden et al., 2018b, Mzyk et al., 2018). Plasma half-lives are generally prolonged for calves and mastitic cows (Landoni et al., 1995a, Sidhu et al., 2017). Absorption has been shown to be delayed with concurrent administration of enrofloxacin in one study, resulting in an overall greater area under the curve (AUC) and peak concentration for flunixin (Abo-El-Sooud and Al-Anati, 2011). However, another study found no changes in flunixin pharmacokinetics with concurrent enrofloxacin administration (Rantala et al., 2002). Various authors have shown that extravascular administration alters pharmacokinetic parameters. Kissell et al. (2012) reported excellent bioavailability (84.5 and 104.2%) and prolonged plasma elimination half-lives (4.48 and 5.39 h) for intramuscular and subcutaneous administration, respectively, compared to an elimination half-life of 3.4 h after intravenous administration. Smith et al. (2015) also reported prolonged plasma half-lives in intramuscularly-dosed cows (14.2-15.5 h intramuscular versus 5.6-11.6 h intravenous). On the contrary, Shelver et al. (2013) found similar plasma elimination half-lives following a single dose of flunixin administered intravenously or subcutaneously (4.7 and 4.5 h,

respectively). The prolonged elimination half-lives in the Smith study could have been due to the treatment regimen or the improved assay sensitivity, resulting in detection of a second terminal elimination phase. In a study investigating the pharmacokinetics of approved flunixin doses (2.2 mg/kg intravenously once daily for 3 days) in veal calves, Kissell et al. (2016) reported an extended plasma elimination half-life of 12.9 h. Kleinhenz et al. (2016, 2018a, 2018b) found the half-life of multiple transdermal flunixin doses in dairy cows was shorter than that following a single administration in calves (5.2 h for multiple doses compared to 6.4-9.3 h after single administration in young calves and 13.2 h in older calves). The prolonged elimination in calves is due to the lower activity of phase I and II enzymes in young animals, leading to reduced clearance. Clearance is also reduced for cows with mastitis, as demonstrated by Kissell et al. (2015) in a study comparing healthy and mastitic cows receiving a single dose of flunixin at 2.2 mg/kg intravenously. The authors postulated this could be due to reduced hepatic metabolism or alterations in protein binding.

Reported half-lives of flunixin in liver and kidney are 34 to 36 h (up to 115 h) and 29 to 32 h (up to 52 h) in nonlactating adult cattle, 28 to 30 h and 21-28 h in lactating cows, and 127.25 and 152.74 in calves (Kissell et al., 2016, Mzyk et al., 2017, Sidhu et al., 2017). Damian et al. (1997) noted a report of liver and kidney flunixin residues of roughly 0.05 µg/mL 5 d after treatment with 2.2 mg/kg intravenous flunixin. Kissell et al. (2016) reported that flunixin was detectable in liver, muscle, and kidney of veal calves through 5 d after last administration of a normal label dose (2.2 mg/kg flunixin intravenously once daily for 3 d). The highest concentrations were reported in liver, with peak concentrations of 3.19 µg/mL observed 1 d following administration. The liver concentrations on day 5 were unexpectedly higher than on day 4, but the authors observed this could have been due to individual metabolic variation in the

sampling groups. The authors estimated a 10-day depletion time would be necessary for liver concentrations to drop below the tolerance set for approved classes. However, since there is zero tolerance for flunixin in veal calves, the authors also reported the time for liver concentrations to be undetectable, which was 13 d. In a physiologically-based pharmacokinetic (PBPK) model for flunixin in plasma, liver, and milk after various routes of administration, Leavens et al. (2014) reported that route of administration did not affect days to reach tolerance in liver, with an upper limit of uncertainty for WDI in liver of 9 days, regardless of route. On the contrary, Chou et al. (2022) used a PBPK model to predict a meat WDI following administration of labeled doses given intramuscularly; they estimated an 8-day WDI to allow liver residues to deplete to tolerance. Shelver et al. (2016a) investigated the muscle residues in cows treated with labeled doses of flunixin given on-label or intramuscularly (extra-label). They found that residues were higher for cows given intramuscular flunixin and observed the highest concentrations in tongue, heart, and tail muscles. Smith et al. (2015) described flunixin residues in cows administered label doses when given intravenously or intramuscularly and found that route had a significant effect on residue concentration only for injection site. Wu et al. (2025) estimated WDI following labeled and extra-label uses of flunixin transdermal using a PBPK model: they estimated WDI of 1 d and 2 d for liver and muscle after a single dose and 2 d for both liver and muscle after multiple doses. This is shorter than the WDT of 8 d following labeled transdermal flunixin application. In such cases, following ELDU of transdermal flunixin, the WDI is the sum of the labeled WDT plus an additional day. When evaluating the effect of changes in flunixin metabolism due to disease, Lin et al. (2016) reported that disease decreased marker to parent drug ratios and prolonged WDI estimates. The authors suggested that in light of estimations that accounted for pharmacokinetic variability during disease, that the WDT for flunixin should be

prolonged by 3 d. By incorporating the additional variability of diverse plasma pharmacokinetic data in a liver residue population pharmacokinetic (PK) model, Wu et al. (2013) found that WDI estimates were longer than if only including liver data. For inquiries regarding flunixin in dairy heifer, beef steer, and veal calves, FARAD recommends a 14 d WDI (Mzyk et al., 2017). If given extravascularly, FARAD recommends a WDI for meat of 10 d for single labeled doses and up to 60 d for multiple doses.

Jones et al. (2014) investigated the potential for ante-mortem tests to predict violative liver flunixin residuess in heifers. A lateral flow assay was adapted to urine and showed correlation with liver values, leading the authors to suggest that it may be a useful tool to predict animals with violative samples before slaughter. Similarly, Shelver et al. (2016b) found that the ratio of urinary flunixin to 5-OH-flunixin was useful for predicting metabolic alterations that would result in violative liver residues. To the authors' knowledge, no antemortem tests are currently in common use.

The reported apparent half-life of flunixin in milk after intravenous administration ranges from 6.7 to 27 h (Sidhu et al., 2017). Following labeled doses given intravenously or intramuscularly, Smith et al. (2015) observed violative milk residues in 6 of 20 cows at 36 h (the labeled WDT). When given for an additional 2 d beyond the label (2.2 mg/kg intravenously every 24 h for 5 d), Daeseleire et al. (2003) detected flunixin and 5-OH-flunixin in milk for 48 h after administration (up to 0.9 and 2.6 µg/kg), but did not sample after that time. Gorden et al. (2019) administered cows transdermal flunixin in an extra-label manner (3.3 mg/kg once daily for 2 doses) and calculated a terminal milk half-life for flunixin of 20.71 h and 5-OH-flunixin of 22.6 h. Maximum concentrations of flunixin and 5-OH-flunixin (0.01 and 0.061 µg/mL, respectively) were observed at 8 h following the last dose. From their results, they calculated a

WDI of 96 h. Kissell et al. (2012) evaluated the effect of route of administration on flunixin milk

residues in a crossover study and found that extra-label routes (intramuscular and subcutaneous)

prolonged the period of 5-OH-flunixin detection in milk. They also reported that healthy cows

with lower milk yields had reduced flunixin milk clearance compared to high-yielding cows. In a

study comparing a single labeled dose of flunixin to healthy cows and those with mastitis, Kissell

et al. (2015) reported that 8 out of 10 mastitic cows had violative 5-OH-flunixin milk residues at

36 h (the labeled WDT), with 3 of 10 mastitic cows still having quantifiable flunixin parent

compound at 60 h. Based on their results, the authors pointed out the limitations of establishing

WDT using study populations of healthy animals. The prolonged residues they observed may

have been due to decreased whole-body clearance or decreased milk production secondary to

disease. In the PBPK model developed by Leavens et al. (2014), they reported that route of

administration prolonged milk residues.

Ibuprofen

Ibuprofen is a member of the 2-arylpropionate group (Lees, 2018). It is not approved for

veterinary species in the United States, but there are a number of approved products for humans.

The plasma half-lives of ibuprofen in cattle are approximately 1.5 to 3.3 h after

intravenous administration and 3.6 h after intramuscular administration (DeGraves et al., 1993,

Raut et al., 1993). Despite high bioavailability (79 to 112%), peak serum concentrations were

lower after oral compared to intravenous administration, with a mean residence time (MRT) of

4.26 h for oral delivery (DeGraves et al., 1993). Ibuprofen is highly protein bound, like other

NSAID.

DeGraves et al. (1993) evaluated the pharmacokinetics of ibuprofen in lactating cows

following a single dose at 25 mg/kg intravenously or orally. The authors reported a milk

absorption half-life of 0.22 h, MRT of 0.95 h, and a milk to plasma AUC ratio of 0.0029 following intravenous administration. Milk concentrations peaked (0.65 µg/mL) at roughly 25 minutes and were undetectable by 240 minutes. Ibuprofen was detectable but less than the limit of quantification (LOQ) in milk after oral administration.

No data regarding tissue distribution were available in the literature. The lack of tissue data severely restricts the ability to estimate a meat WDI for this drug. Per AMDUCA guidelines, if there is insufficient data to estimate a WDI, the drug should not be administered.

Indomethacin

Indomethacin is an indoline NSAID that is approved for human use in the United States (Lees, 2018).

Following 10 mg/kg indomethacin administered intravenously in 3 mo calves, the elimination half-life was 5.3 h and the volume of distribution was 12.2 h (Cristofol et al., 1996).

No data regarding tissue distribution were available in the literature. The lack of tissue data severely restricts the ability to estimate a WDI for this drug. Per AMDUCA guidelines, if there is insufficient data to estimate a WDI, the drug should not be administered.

Ketoprofen

Ketoprofen is a 2-arylpropionate NSAID and is labeled for subcutaneous administration for control of fever associated with bovine respiratory disease in beef heifers, beef steers, beef calves 2 months of age and older, beef bulls, replacement dairy heifers and dairy bulls (Lees, 2018). As no milk WDT has been established nor a meat WDT for veal calves or calves less than 2 mo, it is not for use in lactating dairy cattle, dairy calves or veal calves. The WDT for approved classes is 2 d (48 h). Ketoprofen is reportedly less irritating than flunixin or phenylbutazone when administered intramuscularly (Smith, 2013).

Ketoprofen is supplied as a racemic mixture of *R*-(−) and *S*-(+) enantiomers. An initial report by Landoni et al. (1995b) found no difference in the pharmacokinetics of the enantiomers in Holstein calves administered a single dose of racemic mixture intravenously, with both enantiomers having an elimination half-life of 0.42 h. In those calves, exudate concentrations persisted longer than plasma concentrations, exhibiting rapid distribution and slow clearance from inflammatory sites. However, in subsequent studies, ketoprofen has been shown to have enantioselective pharmacokinetics. One study in calves and adult dairy cows reported rapid, unidirectional conversion of *R*-(−) to *S*-(+)-ketoprofen (Igarza et al., 2002). The authors of that study reported inversion to be more efficient in preruminant calves (~50%) compared to adult dairy cows (26-33%). In another study in calves administered one of the enantiomers, Landoni et al. (1995) also observed unidirectional conversion of the *R*-(−) to *S*-(+) enantiomer after intravenous administration. Those authors reported 3-fold higher concentrations of *R*-(−)-ketoprofen in exudate compared to *S*-(+)-ketoprofen, but found that the elimination half-life was longer for *S*-(+)-ketoprofen [2.19 vs. 1.30 h for *S*-(+) vs. *R*-(−)]. Enantioselective pharmacokinetics are demonstrated after intramuscular administration as well, with *S*-(+)-ketoprofen having increased AUC, peak concentrations, and time to peak concentrations and decreased clearance and volume of distribution compared to *R*-(−)-ketoprofen (Plessers et al., 2015). Elimination half-life was similar for both enantiomers (2.45-3.0 h). After a single dose in lactating cows, the plasma elimination half-life was approximately 0.5 h (DeGraves et al., 1996). A recent study screened potential transdermal formulations and reported on the comparative pharmacokinetics of different routes of administration (Mills et al., 2022). Ketoprofen was detected approximately 10 minutes after transdermal and intramuscular administration. Transdermal administration resulted in a maximum concentration of 20 µg/mL at roughly 2 h.

Dose corrected values for maximum concentration and AUC revealed higher values for intramuscular compared to the transdermal product. Bioavailability following intramuscular administration was 98%, compared to 50% for transdermal administration. The elimination half-lives of the intramuscular and transdermal products were similar (157 vs. 148 min) and slightly longer than the intravenous route (110 min). In a study comparing the pharmacokinetics of intramuscular and intravenous ketoprofen, Singh et al. (2014) reported intramuscular ketoprofen bioavailability of 77% and a slightly longer elimination half-life compared to intravenous administration (3.4 vs. 1.55 h for intramuscular and intravenous, respectively). Flip-flop pharmacokinetics have been reported for ketoprofen when administered in the combination product containing tulathromycin, with authors reporting a prolonged half-life, lower maximum concentrations, and increased AUC of ketoprofen compared to ketoprofen alone (De Koster et al., 2022).

In lactating cows receiving a labeled dose (3.3 mg/kg once daily for 3 days) of ketoprofen given intravenously, ketoprofen was undetected in milk the day after final administration (Daeseleire et al., 2003). The cows in that study were milked twice daily and ketoprofen was detectable at the first milking (~12 h) after final administration. After a single intravenous injection of ketoprofen, milk was detectable (but below LOQ) in 4 of 6 cows up to 120 min after administration (DeGraves et al., 1996). In that single-dose study, cows were not milked out each time, so there is potential for skewed results based on collection of foremilk vs. whole milk. Although these studies suggest that ketoprofen does not transfer significantly into milk, alterations due to route of administration or disease (e.g. mastitis or other inflammatory processes) could alter milk concentrations.

Meloxicam

Meloxicam is a part of the oxicam group of NSAIDs (Lees, 2018). It is approved for dogs and cats in the United States; human generic tablets are also available.

Following oral administration, meloxicam has a plasma terminal half-life of 20-40 h in calves (Coetzee et al., 2009, Allen et al., 2013, Coetzee et al., 2014, Coetzee et al., 2015). After intravenous administration of similar doses, elimination half-life was 22 h (Coetzee et al., 2012). When comparing preruminant to ruminating calves, Mosher et al. (2012) found that preruminant calves receiving meloxicam through milk replacer (bottle) had faster drug absorption than ruminant calves receiving meloxicam by oral gavage and had higher volume of distribution than either ruminant or preruminant calves receiving meloxicam by oral gavage. Notably, those authors observed that one preruminant calf had an elimination half-life of 9.73 h, compared to mean values ranging from 30 to 40 h in the different study groups; this calf was noted to be the only red Holstein in the study. A more recent study in preruminant dairy calves reported elimination half-lives of approximately 80 h for intravenous and 85 h for subcutaneous and oral administration (Jokela et al., 2024). The animals in that study were younger (less than 3 weeks) and had decreased clearance compared to the older calves from the other studies. The half-life in postweaning calves and adult dairy cows has been reported to be 11.9 to 16.7 h, even in lactating dairy cows receiving a single, high dose (30 mg/kg) of oral meloxicam (Glynn et al., 2013, Malreddy et al., 2013, Gorden et al., 2018a, Fritz et al., 2023, Mzyk et al., 2023). Gorden et al. (2018a) reported that postpartum cattle had higher peak concentrations, AUC, MRT and a delayed time to maximum concentration compared to mid-lactation cows, despite similar elimination half-lives. The authors calculated a relative bioavailability for postpartum cows compared to mid-lactation cows of 210% due to lower volume of distribution and clearance in postpartum animals. In a similar study by Warner et al. (2020) that included an intravenous

comparison, the authors also found an increased relative bioavailability due to decreased clearance, thereby increasing AUC and prolonging the elimination half-life. Mzyk et al. (2023) observed longer elimination half-lives in postpartum (12.9 h) compared to mid-lactation (9.4 h) cows following multiple doses of oral meloxicam. The markedly longer half-life in the calf studies is likely due to the age differences (and thus metabolism and clearance abilities) of the different study populations. Shock et al. (2019) and Meléndez et al. (2019) reported higher AUC, time to maximum concentration, clearance, and volume of distribution for oral versus subcutaneous meloxicam; however, the oral dose in both studies was twice the subcutaneous dose. Even with increased dose, the elimination half-life should theoretically be the same, as was the case for the report by Meléndez et al. (2019). However, Shock et al. (2019) reported a longer elimination half-life for the oral meloxicam group. The authors did not provide an explanation for this observation; it is possible that the oral formulation used (suspension) had markedly different absorption kinetics than the tablet forms used in other studies. Notably, Shock et al. (2019) also found that first lactation cows had higher maximum concentration and AUC than multiparous animals. More recently, a novel microneedle patch has been evaluated for meloxicam delivery in calves (Castilla-Casadiego et al., 2022). In a preliminary study comparing the patch (2.5 and 5 mg/kg treatments) to oral meloxicam (1 mg/kg), maximum concentrations occurred at a similar time (24-48 h) but were much lower for the microneedle patches (0.1 to 0.17 ng/mL for the patches versus 1,600 ng/mL for oral meloxicam).

After repeated oral administration of meloxicam in calves, Coetzee et al. (2015) observed the highest tissue concentrations in liver and kidney 5 d after final treatment which subsequently became undetectable at day 15. In adipose tissue, meloxicam was not quantifiable at 15 d but was detectable at trace concentrations 19 d after final administration. Current FARAD

recommendations are 21 d for a single dose up to 1 mg/kg and 30 d for multiple doses in calves (Mzyk et al., 2017).

The milk elimination half-life of meloxicam has been reported to be 10.4 h in mid-lactation cows, with peak concentrations of 0.41 µg/mL occurring at 9.3 h (Malreddy et al., 2013). In that study, milk concentrations were below the LOQ (10 ng/mL) within 80 h. The authors reported a relatively low excretion ratio into milk (milk AUC to plasma AUC ratio: 0.14). Gorden et al. (2018a) did not detect meloxicam in mid-lactation cows at 120 h, but meloxicam was still detectable at 144 h in postpartum cows; the LOD was 1 ng/mL. Similarly, Mzyk et al. (2023) observed a numerically longer milk elimination half-life in postpartum cows compared to mid-lactation cows receiving multiple doses of meloxicam orally (10.7 vs. 9.6 h). In a study evaluating meloxicam oral suspension, Nagel et al. (2016) reported milk meloxicam concentrations were below LOQ in all animals by 120 h and were well below the maximum residue limits for Canada and the EU by 72 h. The milk withhold time in the EU is 5 d; in the absence of a WDT in the United States, any detection is considered violative (Smith, 2013). Fritz et al. (2023) reported on the milk pharmacokinetics after a single, 30 mg/kg oral dose of meloxicam. Maximum concentrations (33.4 µg/mL) were reached at 23.7 h. The milk elimination half-life was 12.2 h.

Phenylbutazone

Phenylbutazone is a pyrazolone NSAID and is approved for use in dogs and horses in the United States (Lees, 2018). Because of concerns for severe blood dyscrasias in humans, any use in lactating dairy cattle is prohibited by the FDA, so milk residue data will not be discussed in this report (Smith, 2013). As lactation can impact the plasma pharmacokinetics of drugs, only studies evaluating non-lactating animals will be referenced. Due to regulatory concerns with

phenylbutazone and availability of an approved NSAID (flunixin) or others with shorter half-lives than phenylbutazone, FARAD strongly discourages ELDU of phenylbutazone in any class of cattle.

The plasma elimination half-life of phenylbutazone in calves has been reported to be approximately 53 h after intravenous and 58 h after oral administration and has limited extravascular distribution (Arifah and Lees, 2002). Like other drugs, phenylbutazone half-life is age dependent, ranging from 34 h in beef steers to 62 h in bulls to 207 h in neonatal calves (Eberhardson et al., 1979, Lees et al., 1988, Williams et al., 1990, De Veau et al., 2002). Phenylbutazone bioavailability is approximately 70% after oral administration and 89% after intramuscular administration, consistent with reports of higher AUC for intramuscular administration (Eberhardson et al., 1979, Lees et al., 1988, Williams et al., 1990). However, intramuscular injection is associated with severe reactions and prolonged residues.

Considering concerns for potential contamination of untreated animals with phenylbutazone, a few studies have examined possible causes. Cattle sharing feed bunks or cohoused with treated animals, even after cessation of treatment, have detectable phenylbutazone in plasma (Fodey et al., 2015). Barnes et al. (2017) found that cattle grazing pasture formerly contaminated by a phenylbutazone-treated bull had detectable residues. The authors calculated the lowest dose required to obtain detectable plasma residues was 0.04 µg/kg. *In utero* and lactational transfer of phenylbutazone to calves have been reported, warranting extreme caution if considering use of phenylbutazone in gestating beef heifers or beef cows (Chamberlain et al., 2003).

Tolfenamic acid

Tolfenamic is a member of the anthranilic acid class (Lees, 2018). There is no veterinary label in the United States; it is approved for veterinary use in Canada and the EU. There is also no human approval for tolfenamic acid in the United States. Thus, ELDU (in this case, any use) in the United States is illegal. However, considering that it is approved in Canada, it is possible that this drug is still being procured and used extra-label in the United States, so a brief summary will be provided. It should be noted that even in the EU and Canada, extravascular administration of this drug is not permitted (Smith, 2013).

The elimination half-life of tolfenamic acid after intramuscular administration in ruminating calves is 6.7-13.5 h (Lees et al., 1998, Sidhu et al., 2005). Lees et al. (1998) reported longer estimates for higher doses (4 and 8 mg/kg vs. 2 mg/kg); however, other parameters indicated linear pharmacokinetics. The authors suggested that the addition of later timepoints with detectable concentrations improved the accuracy of the estimates for the higher (4 and 8 mg/kg) doses and that enterohepatic recycling or flip-flop pharmacokinetics may be playing a role in the long half-life. Tolfenamic acid appeared at lower concentrations and later in exudate and transudate compared to plasma. Two studies have reported surprisingly large volumes of distribution (1.2 to 3.2 L/kg), considering the high level of protein binding typical of NSAIDs (Lees et al., 1998, Sidhu et al., 2005). Sidhu et al. (2005) found that while concurrent administration of marbofloxacin did not alter the elimination half-life of tolfenamic acid in calves, it did increase clearance, volume of distribution, and the absorption rate constant and decreased maximum concentration, time to maximum concentration, and AUC. Contrary to the study by Lees et al. (1998), Sidhu et al. (2005) found tolfenamic acid concentrations were higher in exudate and transudate than in serum from 24 to 72 h after administration. Pharmacokinetic analysis revealed peak concentrations were reached at 17 h and were similar in exudate and

transudate. Exudate and transudate MRT were prolonged compared to serum (22.9 to 28.3 h in exudate and transudate vs. 10.6 to 11 h in serum).

FARAD has previously recommended a meat WDI of 7 d and milk WDI of 1 d for a single intravenous dose (Smith, 2013).

Glucocorticoids

Dexamethasone

Dexamethasone is a glucocorticoid labeled for use in all classes of beef and dairy cattle and calves for treatment of primary bovine ketosis, as an anti-inflammatory agent, and for supportive therapy for management, inflammatory or stress conditions. Approved dexamethasone labels encompass multiple routes of administration (intravenous, intramuscular, oral) and state that dosages may be repeated if necessary. One of the only label stipulations is an exclusion of veal calves for extravascular dosing and a recommended dose range (5 to 20 mg, depending on the route). There is a zero WDT for meat and milk; however, a WDT has not been established for preruminating (veal) calves. A combination dexamethasone-trichlormethiazide (diuretic) product is labeled for oral administration in dairy cattle for treatment of udder edema with a milk WDT of 72 h; treatment duration is limited to 3 d.

Although the dexamethasone label allows for considerable flexibility in treatment regimens, more recent information regarding residue kinetics has been published and will be discussed here. The use of glucocorticoids for growth promotion has been the focus of a large body of literature seeking to identify early indicators of this illicit use. Much of the residue data comes from these publications.

A report in older calves (average weight 200 kg) administered a single intravenous dose of dexamethasone sodium phosphate at 40 µg/kg observed a plasma half-life of approximately 9

h (Cherlet et al., 2005). Tainturier et al. (1982) reported a plasma half-life of 4.5 h in lactating cows administered dexamethasone at 0.1 mg/kg intravenously. A study by Fairclough et al. (1981) evaluated the plasma and milk concentrations of dexamethasone in different classes of dairy cattle using long- and short-acting formulations. A short-acting (trimethyl acetate) and long-acting (tributyl acetate) ester demonstrated fairly similar peak plasma concentrations (0.5 and 0.7 to 1.1 ng/mL, respectively). Peak concentrations of the short-acting formulation occurred on day 3 and were undetectable by 8-14 d. The long-acting formulation reached peak concentrations between 1 and 7 d following treatment and was undetectable by 14 d.

In adult Charolais bulls receiving dexamethasone or a dexamethasone-clenbuterol combination therapy, Biancotto et al. (2013) described undetectable concentrations in urine by 3 d of withdrawal. Following a 2-day withdrawal, dexamethasone concentrations were highest in liver (3.66 ng/g) and below the LOQ in muscle. In a group euthanized after 7 d, dexamethasone was below LOQ in all tissues. Similarly, in Charolais bulls receiving a combination of dexamethasone sodium phosphate and prednisolone acetate, dexamethasone was undetected after 6 d in urine, whereas there were trace concentrations in liver (Cannizzo et al., 2011). In a more traditional depletion study, older calves (weighing approximately 200 kg) were administered a single intravenous dose of dexamethasone sodium phosphate at 40 µg/kg and euthanized at 4 timepoints (Cherlet et al., 2005). Dexamethasone concentrations were lowest in muscle, with peak concentrations of 5.1 ng/g on day 1; by day 4, concentrations were below the LOQ. Liver had the highest concentrations of dexamethasone (200 ng/g), also 1 d after treatment; by day 8, dexamethasone was below the LOQ. Kidney had intermediate concentrations (111 ng/g peak concentration on day 1), but still had detectable concentrations 8 d after treatment. Half-lives for muscle, liver, and kidney were 0.57, 0.84, and 0.66 d, respectively. Van Den Hauwe et al. (2003)

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investigated residues of dexamethasone and flumethasone in calves with respiratory infections. Calves in the dexamethasone group received a product containing a sodium phosphate and phenylpropionate ester (total dexamethasone dose of 60 µg/kg) and were euthanized after 72 h. The authors reported the highest concentrations in liver (3.1 to 32.8 ng/g) and the lowest concentrations in muscle (0.8 to 4.5 ng/g). Chicoine et al. (2024) performed a depletion study in beef cattle, administering dexamethasone sodium phosphate at 50 µg/kg intramuscularly for 3 days and euthanizing animals on days 3, 7, 11, and 15. No residues were detected in muscle or adipose tissue. Dexamethasone was only detectable in liver and kidney on day 3. The authors estimated a meat WDI of 7 d.

Tainturier et al. (1982) described the milk pharmacokinetics after intravenous dexamethasone at 0.1 mg/kg. The half-life was 3 h, with peak concentrations of 20.6 ng/mL detected at 30 min; dexamethasone was undetected at 12 h. The milk to plasma concentration ratio was 0.39. Dési et al. (2008) analyzed milk residues in cows treated with 50 mg dexamethasone and 150 mg prednisolone intramuscularly. Dexamethasone was detected in samples taken 2 d after treatment, with concentrations ranging from 2.1 to 3.4 µg/kg; dexamethasone was not detected on day 3. A study by Fairclough et al. (1981) evaluated the plasma and milk concentrations of dexamethasone in different classes of dairy cattle using long- and short-acting formulations. Dexamethasone was undetectable in milk following intramuscular administration of a long-acting formulation by day 14; milk concentrations were 30-50% of plasma concentrations. A tritiated short-acting formulation resulted in low, but detectable concentrations up to 30 d after treatment (0.05 ng/mL). Reding et al. (1997) reported peak dexamethasone milk concentrations of 8.4 ng/mL at 12 h after intramuscular treatment with 60 µg/kg of a combination of esters; concentrations were below 1 ng/mL within 3 d. Chicoine et al.

(2024) conducted a milk depletion study in cows administered dexamethasone sodium phosphate at 50 µg/kg intramuscularly for 3 d. Milk residues of dexamethasone peaked at the first milking (2.6 ng/mL at 12 h) and were detected up to 36 h after treatment; the milk elimination half-life was 6.7 h. There were no differences in milk concentrations across lactational stages.

There is no established tolerance for dexamethasone in cattle tissue or milk in the United States, so any detected residues would be considered violative. However, dexamethasone is considered a low regulatory priority and was removed from the National Residue Program based on its rapid elimination and low risk of causing an adverse event in humans (The Pew Charitable Trusts, 2016).

Flumethasone

Flumethasone is a glucocorticoid approved in horses, dogs, and cats for treatment of rheumatic, allergic, dermatologic, and other glucocorticoid-responsive diseases.

Following administration of 5 or 50 µg/kg flumethasone intramuscularly in calves, serum drug was below the LOD by 48 h in low-dose animals and was still detectable at 45 h after the high-dose (Brambilla et al., 2001). Peak serum concentrations of 9.7 ng/mL were reported at 0.5 h after administration of 50 µg/kg intramuscular flumethasone.

Van den Hauwe et al. (2003) evaluated the tissue concentrations of flumethasone following intramuscular administration at 20 µg/kg, with euthanasia occurring at 24 h. The highest concentrations were recorded in liver (7 to 32 ng/g) and the lowest concentrations were in muscle (0.4 to 0.6 ng/g).

Reding et al. (1997) described the milk depletion of flumethasone after administration of 13.5 µg/kg intramuscularly in Brown Swiss cows. Milk concentrations were 0.1 to 1.2 ng/mL at 12 h and were below the LOD on day 2.

Isoflupredone

Isoflupredone is a mineralocorticoid with some glucocorticoid effects and is approved for treatment of primary bovine ketosis and inflammatory conditions. It may be given via intramuscular or intrasynovial injection at a range of doses and durations. There is a meat WDT of 7 d, but no milk WDT has been established. The approved product (Predef 2X, Zoetis, Inc.) was discontinued by the manufacturer in 2020 and thus is no longer available in the United States. Isoflupredone is often viewed as a safer alternative to dexamethasone, as it has not been reported to be abortifacient.

There is little available research on the pharmacokinetics or depletion of isoflupredone in cattle. Following administration of 20 mg isoflupredone intramuscularly once daily for 3 d to beef heifer calves, Krzeminski et al. (1974) measured residues of the parent (9α-fluoroprednisolone acetate) and metabolite (9α-fluoroprednisolone) compound in tissues. Injection site had the highest concentrations of both parent and metabolite (up to 42,900 ng/g and 18,150 ng/g, respectively). The parent compound was detected sporadically in liver and kidney and the metabolite was detected in a majority of liver and kidney samples through the third day after treatment at concentrations < 45 ng/g. Liver and kidney residues were undetectable by day 7. Adipose and muscle residues were undetectable by day 3 and 1, respectively. Neither compound was detectable in blood. In the same report, the authors administered 20 mg isoflupredone intramuscularly to lactating cows every 12 h for 3 doses and detected no residues in milk.

Prednisolone

Prednisolone is a less potent glucocorticoid than dexamethasone and is labeled for use in dogs in the United States. In the EU, there are a variety of approved intramammary combination products for treatment of mastitis that include prednisolone.

Following intramammary injection with a tritiated prednisolone, prednisolone concentrations in milk were higher in healthy cows compared to those with induced mastitis (peak 0.19 vs. 0.05 % recovery) (Geleta et al., 1984). Similar milk recoveries were reported after intravenous administration. Plasma activity was detected 30 minutes after intramammary administration and peaked within 2 h. When cows were administered a reference product or a novel amoxicillin/sulbactam/prednisolone intramammary infusion, Li et al. (2014) reported prednisolone milk half-lives of 0.9 to 1.6 h, with peak concentrations reached at 2 to 2.5 h. In mastitic cows, prednisolone was not below an established maximum residue limit of 6 ng/mL until 54 h, whereas for healthy cows, residues were below this concentration by 30 h (Liu et al., 2016). Rha et al. (2023) reported milk concentrations below this same maximum residue limit at 12 h and below LOD by 24 h, leading to an estimated WDI of 12 h (first milking timepoint) for cows administered prednisolone intramuscularly.

Industrial hemp

There is currently no approval for IH, IH byproducts, or any individual cannabinoids in veterinary species; thus, there is no established tolerance. The FDA considers detection of any cannabinoid in animal feed or tissue to be an adulterant (FDA, 2019). A variety of global tolerances or toxicity references exist for 9-THC and CBD. The European Food Safety Authority (EFSA) has established a lowest observed adverse effect level (LOAEL) of 36 µg/kg and an acute reference dose (ARfD) of 1 µg/kg for 9-THC (EFSA, 2015, Arcella et al., 2020). Infants were not considered in this assessment. Some scientists believe this ARfD is unnecessarily strict

and does not account for the most recent data on IH in feed products (Skoczinski et al., 2019, Beitzke and Pate, 2021). Other countries have higher thresholds (6-7 µg/kg/d) for equivalents of the United States' acceptable daily intake (Beitzke and Pate, 2021). Because the EFSA recommendation is the strictest, most IH literature in livestock has used their ARfD when calculating human exposure estimates. The Food Standards Agency (FSA) has established an acceptable daily intake of 0.15 mg/kg/d for CBD (Choudhry and Haynes, 2024). While exposure estimates are presented below, the authors are not condoning the use of IH in veterinary species, given the current regulatory status.

Seed products

Hempseed has been the most frequently evaluated IH form, due to its low cannabinoid content and favorable protein content (Kleinhenz et al., 2020b).

Chakrabarty et al. (2022) developed a rapid method of quantifying cannabinoids in biological samples using direct-delivery electrospray ionization mass spectrometry. The authors analyzed samples acquired from a feeding trial investigating inclusion of hempseed cake at 20% of the diet, on a dry matter basis. Animals were fed for 112 days and tissues collected from control and hemp-fed animals. Cannabinolic acid (CBNA), cannabidiolic acid and Δ9-tetrahydrocannabinolic acid (CBDA and THCA, acidic precursors of CBD and 9-THC), CBCA, and CBDVA were consistently detected in plasma at low concentrations (0.1 to 1.7 ng/mL). Multiple acidic cannabinoids [CBNA, CBDA/THCA, CBCA, CBDVA, and cannabigerolic acid (CBGA)] were detected in liver on day 0 of withdrawal, ranging from 1.2 to 12.4 ng/g. On day 1, CBDA/THCA was still detected, but at concentrations below the LOQ. No cannabinoids were detected in kidney or muscle.

Smith et al. (2023) evaluated the cannabinoid residue profile in cattle administered hempseed cake for 111 d. The cannabinoid with the highest average intake values was the isomers cannabidiolic acid and Δ9-tetrahydrocannabinolic acid (CBDA and THCA, acidic precursors of CBD and 9-THC) at 37.9 µg/kg. Average intakes of CBD/9-THC were 6.2 µg/kg. In plasma, no neutral cannabinoids were detected and acidic cannabinoids [cannabinolic acid (CBNA), CBDA/THCA, and cannabidivarinic acid (CBDVA)] were detected only sporadically. Detection of cannabinoids in urine was also sporadic. Overall, the authors concluded that urine or plasma would be poor antemortem biosamples for surveillance testing. No cannabinoids were detected in muscle. In liver, CBNA, CBDA/THCA, cannabichromenic acid (CBCA), and CBDVA were detected on day 0 (first day of withdrawal), with CBDA/THCA being present in the highest concentrations (35.9 ng/g). Only CBNA and CBDA/THCA were detected on day 1, and all cannabinoids were below the LOD on day 4. In kidney on day 0, CBDA/THCA was also present at higher concentrations (4.3 ng/g) compared to the other detected cannabinoids (CBNA, CBCA, CBDVA; CBGA was below the LOQ in 4 animals). Both CBNA and CBDA/THCA were detectable at low concentrations (0.8 ng/g) in all animals on day 4 and in 2 animals on day 8. Adipose had lower concentrations of acidic cannabinoids (only CBDA/THCA and CBCA were detected at 0.7 and 0.6 ng/g) but was the only tissue in which the neutral cannabinoids CBD/9-THC were detected. Peak concentrations of CBD/9-THC were observed on day 0 (10.1 ng/g) and had reduced by about 40% on day 8 (6.3 ng/g). While there was a linear trend for residues to decrease over time, there was not a statistically significant difference in concentrations across timepoints. The authors calculated human exposure estimates for beef fat consumption and found that no group exceeded the EFSA's 1 µg/kg ARfD for 9-THC.

Contrary to Smith et al. (2023), Addo et al. (2023a) found that supplementing dehulled hempseed meal in lieu of canola meal did not result in detectable cannabinoids in urine, milk, or plasma in lactating cows. Only CBD, CBDA, 9-THC, and THCA were evaluated. Intake values for this study were 0 mg/kg 9-THC, 0.0005 mg/kg THCA, 0.023 mg/kg CBD, and 0.027 mg/kg CBDA. The authors reported detectable concentrations of CBD and CBDA in feces (approximately 0.8 µg/g for both cannabinoids), estimating that roughly 63 to 69% and 52 to 63% of ingested CBD and CBDA, respectively, were excreted. In a similar study in non-lactating cows, Addo et al. (2023b) did not detect cannabinoids in plasma, rumen fluid, liver, kidney, muscle, adipose, or urine. As in the study in lactating cows, the authors reported CBD and CBDA in the feces (0.42 to 0.68 and 0.40 to 0.67 µg/g, respectively). The lack of detectable cannabinoids in rumen fluid was unexpected but may have been due to the collection procedure used. Rumenocentesis did not allow for any feed mat to be collected. The reason behind the lack of measurable tissue cannabinoids in the study by Addo et al. (2023b) is unclear. Cannabidiol intake in the Addo study (63 µg/kg) was higher than that reported by Smith (Addo et al., 2023b, Smith et al., 2023). Although the LOD in the Addo study was 10 ng/g, Smith reported liver and adipose concentrations exceeding that value.

Overall, feeding hempseed products results in relatively low concentrations of cannabinoids in biological samples. Liver appears to accumulate and fairly rapidly deplete acidic cannabinoids, whereas neutral cannabinoids accumulate in adipose.

Extracted plant material

Following extraction of CBD and other cannabinoid oils, there is a large amount of extracted plant material generally considered as waste. Depending on the extraction method, this material may be referred to by different terms, including spent hemp biomass (SHB) and IH

ethanol extraction byproduct (IHEEB). Use of this product in livestock feed is particularly attractive, as CBD oil is a primary market in the United States, leading to generation of large amounts of SHB. Irawan et al. (2025b) investigated the distribution and depletion of cannabinoids in Jersey cows fed SHB at 7.5% of the diet. Cows were fed SHB or a control for 28 d intervention period, followed by a 4 wk withdrawal and then a routine dry-off period. Milk was collected throughout the withdrawal period and at 7 d postpartum (mean of 102 d after final feeding). Adipose, liver, and muscle were collected during the withdrawal period. Feces, blood, and urine were collected during the intervention period. Average doses of CBD and 9-THC were 15.6 and 0.81 mg/kg, respectively. Based on calculations of cannabinoid fecal excretion, approximately 64% of total cannabinoids were absorbed, with 9-THC, CBDA, and CBCA having higher absorption and THCA, CBD, and CBC having lower absorption. There was not an obvious pattern in percent absorption between the acidic and neutral cannabinoids or accounting for varying cannabinoid dosages. This suggests that cannabinoids are metabolized in the gastrointestinal tract (by microbial or endogenous enzymes), or active transport systems play a potential role in gastrointestinal absorption, resulting in certain cannabinoids being preferentially absorbed, regardless of dose. A large number of acidic cannabinoids were detected in plasma (CBCA, CBDA, THCA), along with CBD, 9-THC, CBC, CBG, and 11-nor-9-carboxy-tetrahydrocannabinol (THC-acid or THC-COOH), in inactive main metabolite of 9-THC. The authors detected THC-acid at the highest concentrations (4,675 ng/mL), followed by CBDA (approximately 500 ng/mL). On day 1 of withdrawal, cannabinoid concentrations were highest in liver (approximately 15,000 ng/g total cannabinoids, 7,500 ng/g CBDA, 3,500 ng/g THCA and CBCA). Cannabinoids were undetectable in liver on day 28. There was minimal accumulation in muscle tissue, with mean concentrations of roughly 100 ng/g for total cannabinoids, 50 ng/g for

THC-acid, and 25 to 35 ng/g for CBDA and CBCA. As for liver, concentrations of cannabinoids

in muscle were undetectable or at incredibly low concentrations on days 15 (59.1 ng/g total

cannabinoids) and 28 (approximately 10 ng/g or less total cannabinoids). Although adipose

concentrations were less than in liver on day 1, they depleted more slowly. On day 1, total

cannabinoid concentrations were slightly less than 4,000 ng/g, CBDA was approximately 3,000

ng/g, and 9-THC was approximately 400 ng/g. The authors reported very low concentrations of

THC-acid in adipose (60 ng/g) during the feeding period. On day 15, total cannabinoid and CBD

concentrations were approximately 1,800 to 2,000 ng/g and 9-THC concentrations were slightly

reduced. By day 28, the authors reported mean CBD concentrations of 628 ng/g and 9-THC

concentrations of 92 ng/g, although significant variability was noted. Similar to Smith et al.

(2023), this report by Irawan et al. (2025b) demonstrates that neutral cannabinoids preferentially

accumulate in adipose and are slowly eliminated. This slow depletion of cannabinoids from fat

has also been reported in humans and rodents (Johansson et al., 1989, Gunasekaran et al., 2009).

While cannabinoids were still detectable in fat up to 28 d, Irawan et al. (2025b) reported most

cannabinoids were detectable in milk by 12 d of withdrawal. A total of 12 cannabinoids were

initially detected on the last day of feeding, with CBDA and CBD being present in the highest

concentrations (3,265 and 983 ng/mL, respectively). The psychoactive cannabinoid, 9-THC, and

its precursor THCA were detected at 100.1, and 42.0 ng/mL on the last day of feeding. CBN,

CBG, and CBNA were present at low concentrations (less than 10 ng/mL). Additional

cannabinoids (CBGA, CBC, CBCA, and CBDVA) were detected at intermediate concentrations.

Mean cannabinoid transfer to milk was 0.42%, 9-THC transfer was 0.87%, CBD transfer was

approximately 0.4%, and CBDA transfer was approximately 0.6%. These transfer rates are

higher than in other studies that have used Holstein cows, as opposed to Jerseys (Wagner et al.,

2022). Both CBD and CBDA were detected through day 28 of withdrawal (32 and 103 ng/mL on day 12 and 8.6 and 40 ng/mL on day 28, respectively), whereas all other cannabinoids were undetectable. At 7 d postpartum—following a 28-day withdrawal, dry-off period, and calving, during which a control diet was fed—CBD and CBDA were detectable in milk at higher concentrations (45 and 203 ng/mL, respectively) than at day 28 of withdrawal. The authors suggested that this could be due to accumulation of these cannabinoids in fat, followed by subsequent release due to lipolysis that occurs during the energy-intensive postpartum period. Irawan et al. (2025b) calculated consumer exposure assessments and determined that high fat consumer groups < 18 yr would exceed the EFSA's 1 µg/kg ARfD for 9-THC but would not reach the LOAEL of 36 µg/kg.

Wang et al. (2023) studied the effects of IHEEB and wildrye hay in the diets of Holstein cows in a 3 x 3 Latin square design study. In each of three 21-day feeding periods, cows would receive a control diet (0% IHEEB), 6% IHEEB and 1.7% wildrye hay, or 10.8% IHEEB and 4.3% wildrye hay. The IHEEB material had 0.3 mg/g CBD (dry matter basis), whereas 9-THC was below the limit of quantification. The low-IHEEB diet contained 0.02 mg/g CBD and the high-IHEEB diet contained 0.03 mg/g CBD. The authors did not detect CBD or 9-THC in the milk. The LOD and LOQ for milk were 1.5 and 5 ng/mL, respectively.

The IHEEB diets used by Wang et al. (2023) contained considerably less cannabinoids than the SHB used by Irawan et al. (2025b), despite being used at similar or higher levels of dietary inclusion. This difference in cannabinoid content speaks to the variability in extraction methods and can explain the lack of residues observed in cows fed IHEEB.

Unextracted plant material

Various groups have looked at feeding a variety of unextracted hemp products, including IH inflorescence, silage, and leaves. While these represent a disparate group of plant and potential dietary components, they are grouped together due to their higher concentrations of cannabinoids compared to hempseed materials or extracted plant products.

In the first report of IH administration in cattle, Kleinhenz et al. (2020a) administered Holstein steers a single dose of IH inflorescence at 5.4 mg/kg CBDA. This dose was based off the labeled CBD dose for humans suffering from certain severe forms of epilepsy. Doses of THCA, CBDVA, and CBCA were 0.6, 0.02, and 0.5 mg/kg, respectively. The IH cultivar used in the study was selected for its high CBDA content. The authors observed 4 acidic cannabinoids in all cattle: CBDA, THCA, CBDVA, and CBCA. The neutral cannabinoid CBD was additionally detected in 4 total samples from 2 animals. Plasma CBDA reached the highest concentrations, with peak concentrations of 72.7 ng/mL (range 36.5 to 177 ng/mL) occurring at 11.8 h. The maximum concentrations of THCA, CBCA, and CBDVA were 12.1, 12.3, and 13.1 ng/mL, which were reached at 25.2, 23.2, and 13.6 h, respectively. The apparent half-life of CBDA was 14.1 h. The MRT extrapolated to infinity was 30.6 h. Volume of distribution was quite low at 39.7 L/kg, indicating limited distribution, potentially due to high levels of protein binding. In humans, plasma 9-THC is highly protein bound (95-99%) (Grotenhermen, 2003).

In a subsequent multiple dose study, Kleinhenz et al. (2022) administered Holstein steers IH inflorescence at a target dose of 5.5 mg/kg once daily for 14 days. They detected CBDA, THCA, CBDVA, CBCA, and CBD, as in the previous study, but also detected CBGA. Pharmacokinetic parameters for CBDA were fairly similar to the single-dose study, with peak concentrations reached at 16.7 h and a terminal half-life of 15.3 h. Peak concentrations in this study were considerably lower than the previous study, at 22.1 ng/mL. Differences in

cannabinoid profiles and concentrations between the studies are likely due in part to IH cultivar differences and potentially also to variability in individual animals' metabolic capacity. Minimum CBDA concentrations of 12.3 ng/mL were observed at 9.3 h after initial feeding. The authors calculated an accumulation index of 1.52, indicating that steady state concentrations were approximately 150% greater than concentrations following the first dose. Steady state concentrations were reached in roughly 7 days.

Fruge et al. (2025) investigated the depletion profile of cannabinoids in cattle fed IH leaves. Five cannulated steers were fed hay and IH leaves at a dose of 3.9 mg/kg CBDA for 14 d, then allowed to graze pasture (no IH exposure) for 45 d. Rumen fluid, plasma, feces, and adipose tissue were collected. CBDA was the highest cannabinoid measured in rumen fluid, but other acidic cannabinoids and CBD and 9-THC were detected. In plasma, CBDA was detectable by 2 h after administration and peaked at 72 h; concentrations were approximately 104 ng/mL for the first three days of feeding. The cannabinoids THCA, CBDVA, CBD, THCVA, and CBD-7-acid, a primary metabolite of CBD in humans, were also detected (Kleinhenz et al., 2020a, Fruge et al., 2025). They did not detect 9-THC. By 96 h after the last day of feeding, CBDA had declined to 24.8 ng/mL, CBD to 0.88 ng/mL, and THCA to 2.82 ng/mL. In adipose tissue, CBDA was only detected for 1 day following the last dose (35 ng/g). On the contrary, CBD and THC were detectable throughout the 45-day withdrawal period. After 1 d, 2 wk, and 6 wk following the last dose, adipose CBD concentrations were 247, 130, and 43 ng/g, respectively. After 1 d, 2 wk, 4 wk, and 6 wk following the last dose, adipose 9-THC concentrations were 14.9, 9.7, 5.8, and 3.4 ng/g, respectively. (Concentrations of CBD at 4 wk were not specifically reported but appeared to be intermediate to the 2 and 6 wk sample concentrations upon visual inspection of the figure.) Cannabinoids were detected at high concentrations in feces, with CBDA reaching the highest

concentrations. Numerous cannabinoids were detected in feces and the authors noted that there was a large amount of variability in fecal cannabinoid concentrations among the animals, suggesting that individual metabolic differences were responsible.

Schwerdtfeger et al. (2025) also evaluated the effects of IH leaves, using lactating Holsteins in a two-period crossover study. First-lactation cows were administered a total mixed ration with the addition of either 7.4% IH leaves (dry matter basis) or 3.5% soybean meal for 21 days. Following a 2-week washout period (no IH or soy in the ration), animals received the opposite diet. Plasma was collected once at 14 d during each feeding period and milk was collected from a consecutive evening and morning milking. The IH cultivar used (Santhica 27), is known for its low 9-THC content. Average cannabinoid doses for hemp-treated animals were 3.9 mg/kg CBD, 0.37 mg/kg 9-THC, and 0.21 mg/kg THCA. No CBDA was reported in the IH leaf analysis. Unlike previous reports, the authors observed highest concentrations of CBGA in plasma (181 ng/mL), followed by much lower concentrations of CBDA (5.9 ng/mL). Other cannabinoids (CBC, CBD, CBG, 9-THC, and THCA) were all detected at concentrations < 0.5 ng/mL, besides CBG (1.6 ng/mL). Concentrations of 9-THC were the lowest, at 0.003 ng/mL. Interestingly, the authors reported that three control cows had positive concentrations of CBDA and CBGA (0.003 and 0.038 ng/mL, respectively) and one control cow had detectable concentrations of 9-THC (0.01 ng/mL). The authors did not report whether the control animals with positive cannabinoid concentrations had received the IH treatment in the previous period. It is possible that these detectable concentrations were due to the prior exposure, but could also represent contamination from treated animals, as has been reported for NSAIDs (Popot et al., 2007, Hairgrove et al., 2019, Bates et al., 2020, Fritz et al., 2022). Milk concentrations followed a slightly different pattern, with CBGA and CBD being present in the highest concentrations (1.5

and 1.2 ng/mL, respectively), whereas other cannabinoids were present at < 1 ng/mL. Contrary to plasma, CBDA was the cannabinoid with the second-lowest concentration in milk, after THCA (0.05 and 0.008 ng/mL, respectively). In general, neutral cannabinoids were represented in higher concentrations in milk, whereas acidic cannabinoids had higher concentrations in plasma. The authors found no significant correlations between plasma and milk cannabinoid concentrations but did find a tendency towards correlation for CBD concentrations.

Wagner et al. (2022) investigated the cannabinoid transfer to milk following hemp silage administration. Holstein cows were fed a hemp-free diet during a 7-day control period, whole-plant hemp silage from a low-cannabinoid variety (termed silage A) during a 7-day adaptation period, followed by a 6-day period of exposure to either low or high hemp inclusion levels of a leaf-flower-seed silage from a high-9-THC variety (silage E). Following this, cows underwent an 8-day depuration period with a hemp-free diet. Milk was collected prior to adaptation and at regular intervals from the end of the adaptation period to the end of the depuration period. Blood and feces were collected at the end of each study period. During the adaptation period, cows received up to 0.09 mg/kg 9-THC, 0.01 mg/kg THCA, and 1.22 mg/kg CBD. During the exposure period, low-hemp cows received an average of 1.64 mg/kg 9-THC, 0.09 mg/kg THCA, and 10.9 mg/kg CBD. High-hemp cows received an average of 3.5 mg/kg 9-THC, 0.2 mg/kg THCA, and 23.2 mg/kg CBD. The authors reported measurable concentrations of 9-THC, THCA, Δ9-tetrahydrocannabivarin (THCV), CBD, CBN, and cannabidivarin (CBDV) in plasma at the end of the adaptation period and during the exposure period. Peak milk concentrations of 9-THC and CBD were 316 and 1,174 µg/kg in the high-hemp group. Four other cannabinoids were also detected: THCA (1.9 µg/kg), THCV (8 µg/kg), CBN (2.5 µg/kg), and CBDV (10.1 µg/kg). Both 9-THC (1.4 and 5.0 µg/kg for low- and high-hemp groups) and CBD (7 and 16.2

µg/kg for low- and high-hemp groups) were still detected on the last day of the depuration period (i.e., after 8 d of withdrawal). A biphasic elimination pattern was observed, with rapid decline in milk concentrations after cessation of feeding hemp silage, and then slower decline over time. The authors calculated a feed to milk transfer rate of 0.2%. A toxicokinetic model revealed fecal excretion of 23 and 36% for 9-THC and CBD, with the remaining 77 and 64% of elimination due to processes such as urinary elimination and metabolism within the gastrointestinal tract. Exposure estimates generated using the milk data from this study revealed the EFSA's 1 µg/kg ARfD for 9-THC was exceeded for several consumer groups during the exposure, but not adaptation, period. Younger demographics had higher estimated levels of exposure. Only infants in the high dairy consumer category exceeded the threshold during the adaptation period (estimated exposure: 1.5 µg/kg 9-THC).

The much higher residues observed by Wagner et al. (2022) compared to other studies involving unextracted hemp leaves or SHB are likely due to diet formulation and cultivar differences (Wagner et al., 2022, Irawan et al., 2025b, Schwerdtfeger et al., 2025). Wagner fed cows a high level of hemp silage using a cultivar with a high 9-THC content, whereas Schwerdtfeger used a low-cannabinoid, low-9-THC cultivar and Irawan used a lower dietary inclusion rate of an extracted product. In addition, a recent study has shown that ensiling increases the proportions and total amounts of neutral cannabinoids in IH material, which could partially explain the high 9-THC and CBD residues reported by Wagner (Klevenhusen et al., 2024).

Cannabidiol oil

While a main attraction of IH in cattle feed is the ability to transform "waste" fibrous plant material into edible tissues products (meat and milk), CBD oil has gained a substantial

interest in canine and equine research. Indeed, the FDA has issued warning letters to companies illegally selling CBD products for these species. Meyer et al. (2022) investigated the plasma pharmacokinetics of CBD following a single oral administration of CBD oil at 5 mg/kg CBD to preruminant calves. Peak concentrations of 0.05 µg/mL were reached at 7.5 h. The elimination half-life was 23 h and MRT was 35 h. Clearance (5.29 L/kg/h) and volume of distribution (176 L/kg) were much higher than the previous reports of CBDA pharmacokinetics (2.0 L/kg/h and 39.7 L/kg, respectively) (Kleinhenz et al., 2020a). The CBD elimination half-life and MRT were increased compared to CBDA values. Meyer et al. (2022) reported an AUC value for CBD approximately one-third that of the value for CBDA (0.95 h×µg/mL compared to 2.7 h×µg/mL). This overall lower AUC for CBD compared to CBDA is likely due to a slightly lower maximum concentration and faster clearance. The disparity between the lower AUC but higher MRT for CBD compared to CBDA could be explained by the increased volume of distribution for CBD. Considering that CBD is more lipophilic than its acidic precursor, CBDA, this increase in volume of distribution is not surprising. As discussed for the previous studies evaluating cannabinoid tissue residues after IH exposure, neutral cannabinoids, such as CBD, appear to accumulate in adipose tissue. Therefore, it is also possible that the prolonged MRT reported by Meyer et al. (2022) is due to continued release of CBD from depot sites (Johansson et al., 1989, Gunasekaran et al., 2009, Smith et al., 2023, Fruge et al., 2025, Irawan et al., 2025b). The results from this CBD oil study indicate that CBD is poorly bioavailable following oral administration, with large variability between animals.

DISCUSSION

With the recent addition of the analgesic label to the flunixin approval (for both intravenous and transdermal formulations), justifying ELDU of other compounds for either anti-

inflammatory or analgesic purposes is challenging. However, certain patient and disease-specific factors may contraindicate use of flunixin. While there are studies evaluating anti-inflammatory use in certain painful or stressful management procedures (e.g. dehorning, castration, branding), there is much still to learn regarding the most efficacious means of treating pain or inflammation due to physiologic processes (e.g. calving) and disease states (e.g. neonatal calf diarrhea, lameness, soft-tissue or orthopedic trauma, chronic orthopedic or neuropathic pain). As the current review has indicated, marked differences in pharmacokinetics of NSAIDs in calves warrant further investigation into both the pharmacokinetics and pharmacodynamics of different compounds across animal classes. Ultimately, the drug approval process is expensive and lengthy and additional approved NSAIDs may not become available in the near future.

While there are approved glucocorticoids in the United States, recent literature has demonstrated that labeled WDT may need to be reconsidered. Considering the effects of route of administration, disease, and age that have been discussed throughout this review, it is likely that the broad label indications of dexamethasone may result in prolonged residues. Although considered of low regulatory concern, veterinarians should be mindful of the destination of the animal they are treating and should be transparent with producers about potential residues.

There is relatively little research on the tissue disposition of cannabinoids in cattle following exposure to IH. The available research is diverse with respect to the amount and form of IH included in the diet. In addition, variability between cultivars makes it challenging to directly compare available reports. However, a few themes have consistently arisen. Acidic cannabinoids predominate in plasma, whereas neutral cannabinoids, such as 9-THC and CBD, accumulate preferentially in fat and are excreted into the milk. The current literature provides foundational information to assist regulatory agencies in establishing a toxicity threshold (e.g.

NOAEL, LOAEL, ADI, or ARfD) for 9-THC and CBD. The tentative AAFCO approval for hempseed meal in layer hens has established guidelines for maximum dietary inclusion and maximum 9-THC and CBD content of hempseed meal (Hemp Feed Coalition, 2024). A similar approach could be worthwhile for cattle, as it would be able to be applied to various cultivars and preparation or extraction methods or hemp product types (e.g. seed, inflorescence, leaves, whole plant). Moving forward, studies evaluating different dietary forms within the same animal demographic would be useful for elucidating unique pharmacokinetic effects of the dietary matrix. Ultimately, focusing efforts on IH by-products (hempseed meal or extracted plant material) will be imperative: there is precedence for approval of hempseed meal as a feed ingredient in laying hens and the by-product forms are of the greatest interest from a sustainability perspective.

The purpose of this review has been to summarize the available literature on the pharmacokinetics of unapproved anti-inflammatory drugs in cattle. It is the duty of every veterinarian to familiarize themselves with the requirements for ELDU set forth by AMDUCA and to determine an appropriate WDI, should ELDU be deemed necessary. As mentioned previously, FARAD is an excellent resource for ELDU regulations, recent literature, approved drugs in food animals, and offers invaluable services by providing WDI estimates in response to inquiries. Readers are encouraged to contact FARAD in the case of any concerns or questions regarding ELDU in cattle and other food animals.

Figure 1.1. Flowchart demonstrating the literature search process, exclusion criteria, and results.[1]

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[1] Created in BioRender. Fritz, B. (2025) https://BioRender.com/wvgas7u.

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Chapter 2 - Determination of milk concentrations and pharmacokinetics of salicylic acid following acetylsalicylic acid (aspirin) administration in postpartum dairy cows[2]

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ABSTRACT

The objectives of this descriptive study were to (1) describe the pharmacokinetics of salicylic acid (SA) in the milk and plasma of postpartum dairy cattle following oral administration of acetylsalicylic acid (ASA; aspirin), (2) to estimate a recommended milk withdrawal period for dairy cattle treated with ASA, and (3) to determine the effect of ASA administration on plasma prostaglandin E_2 metabolite (PGEM) concentrations. Primiparous (n = 3) and multiparous (n = 7) postpartum Holstein dairy cows received 2 oral treatments with ASA at 200 mg/kg of body weight, 24 h apart. Concentrations of SA in plasma and milk from 0 h through 120 h after ASA administration were analyzed using ultra performance liquid chromatography triple quadrupole mass spectrometry and a milk withdrawal period was estimated using the United States Food and Drug Administration Milk Discard App in R. Two withdrawal periods were estimated: (1) a whole-herd treatment scenario with no dilution factor and (2) an individual animal treatment scenario with a bulk tank factor included in analysis. Plasma PGEM concentrations in samples from 0 h to 24 h after ASA administration were determined using a commercially available competitive ELISA. Milk SA concentrations were undetected in all cows by 48 h after the last ASA treatment. Secondary peaks were observed in plasma at 58 and 82 h after the last treatment and in milk at 87 h after the last treatment. In the absence of a tolerance for SA in milk, the estimated milk withdrawal periods were (1) 156 h for the whole-herd treatment scenario and (2) 120 h for the individual animal treatment scenario. Plasma PGEM concentrations were reduced compared with baseline for up to 12 h after ASA administration, with the greatest reduction observed at 2 h. Results from this study suggest that the current milk withhold recommendation for dairy cattle administered ASA may need revision to 120 h (5 d) and that ASA administration may mitigate postpartum inflammation through

reduction in prostaglandin production for up to 12 h after treatment. Pharmacokinetic and milk withdrawal data from this study will inform future recommendations for extra-label use of aspirin in postpartum dairy cows. Further research is required to determine the basis for the secondary SA peaks and to elucidate the long-term effects of ASA administration on dairy cow health.

Key words: acetylsalicylic acid, nonsteroidal anti-inflammatory drugs, postpartum, pharmacokinetics

INTRODUCTION

Administration of nonsteroidal anti-inflammatory drugs (NSAID) at calving may reduce inflammation in the transition period and the associated negative effect on milk production (Farney et al., 2013a). Previous work has shown that periparturient administration of sodium salicylate (SS), acetylsalicylic acid (ASA), meloxicam, ketoprofen, or carprofen increases milk yield (Farney et al., 2013b, Stilwell et al., 2014, Carpenter et al., 2016, Kovacevic et al., 2018, Shock et al., 2018, Barragan et al., 2020a). Reported benefits of NSAID administration include decreased SCC, decreased culling rates, increased activity, and increased feeding behavior (Mainau et al., 2014, Stilwell et al., 2014, Shock et al., 2018).

The use of the NSAID, SS, and ASA (aspirin) has also been described in postpartum dairy cows (Farney et al., 2013b, Carpenter et al., 2016, Barragan et al., 2020a). Acetylsalicylic acid and its active metabolite, salicylic acid (SA), inhibit cyclooxygenase-1 and -2, reduce tumor necrosis factor-α (TNF-α) mRNA transcription, and prevent nuclear factor kappa B activation by binding to the protein inhibitor of nuclear factor kappa B kinase subunit beta (Mitchell et al., 1993, Yin et al., 1998, Myers et al., 2010). Acetylsalicylic acid is not currently approved in the United States for use in lactating dairy cattle (Smith et al., 2008). Many authors have reported

increased milk yields after treating postpartum, multiparous cows with ASA, similar to the effects seen with other NSAID (Trevisi and Bertoni, 2008, Farney et al., 2013b, Barragan et al., 2020a). A recent study found that cows treated with ASA had a significantly lower incidence of metritis and a tendency toward lower rates of endometritis (Barragan et al., 2021). The authors also reported a tendency in the ASA group to faster conception rates, a lower incidence of pregnancy losses, and increased conception rates following pregnancy loss compared with untreated cattle.

Although the potential benefits of ASA administration are attractive to producers, milk residues need to be investigated. Currently, no published pharmacokinetic (PK) data are on SA in milk. Though PK data are available for serum salicylate concentrations (Gingerich et al., 1975, Anderson et al., 1979, Coetzee et al., 2007, Kotschwar et al., 2009, Baldridge et al., 2011, Bergamasco et al., 2011), the treatment regimens used were unlike those described in more recent studies. Some of these earlier studies investigated the use of SS through either a single intravenous administration at 50 mg/kg of BW (Coetzee et al., 2007, Kotschwar et al., 2009, Bergamasco et al., 2011) or oral administration through drinking water for 5 d at doses ranging from 13.6–152 mg/kg of BW (Baldridge et al., 2011). Two studies investigated oral administration of ASA, either at 50 mg/kg of BW for one treatment (Coetzee et al., 2007) or at 100 mg/kg of BW per treatment every 12 h for 5 d (Gingerich et al., 1975). More recently, Barragan et al. (2020a, 2020b, 2021) reported that a short course of ASA treatment—200 mg/kg every 24 h for 2 d—has positive effects on milk production and cow metabolic and reproductive well-being. This revised treatment protocol may be more practical and, thus, more likely to be adopted by researchers and producers for use in postpartum cows. In addition to the updated dosing regimen, the improved quality and sensitivity of analytical equipment make PK data

gathered today more accurate. Drug concentration data in plasma and milk are needed to establish the pharmacokinetic profile of SA, to recommend milk withhold times, and to inform future approval decisions and regulations regarding milk withholding periods for lactating dairy cattle. Supplying producers with appropriate, well-founded data on milk residues is critical to maintaining consumer trust and food supply integrity. Minimal pharmacodynamic data are available for dairy cattle following oral administration of ASA. Determining the short- and long-term effects of ASA on dairy cow health would provide producers with information necessary to form optimal postpartum cow health management programs.

The objectives of this study were to (1) describe the pharmacokinetics of SA in the milk and plasma of postpartum dairy cattle following oral administration of aspirin, (2) to establish a recommended milk withdrawal interval (WDI) for dairy cattle treated with ASA at 200 mg/kg twice, 24 h apart, and (3) to determine the effect of ASA administration on plasma concentrations of prostaglandin E_2 metabolites (PGEM) compared with baseline PGEM values.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Kansas State University approved the methods outlined below (protocol number 4432).

Animals, housing, and treatments

In this descriptive study, postpartum Holstein cattle (Bos taurus; n = 10; 3 primiparous, 7 multiparous) of an average weight of 662 kg (554–859 kg) from the Kansas State University Dairy Teaching and Research Center were enrolled between May 2021 until July 2021, within 3 to 27 h after calving. The Dairy Teaching and Research Center houses approximately 300 cows. A common, straw-bedded pen was used to house cows that were within approximately 30 d of calving. To be eligible for enrollment, cows were required to be free of illness; have a lameness

score of ≤2 out of 5 using a visual lameness scoring system (Sprecher et al., 1997); have a dystocia score of 1 out of 5 (Barragan et al., 2020c); and have no history of receiving ASA or other NSAID in the past 30 d. If any cows developed lameness or illness or required other medications during the study period, they were unenrolled. Throughout the enrollment period for each cow, body temperature was measured at least once per day using rectal thermometry and urine ketones were analyzed once per day with ketone reagent strips supplied by the Kansas State Dairy Teaching and Research Center. Farm personnel administered a single bolus of BoviKalc (Boehringer Ingelheim Vetmedica) to cows at the time of calving. Eligible cows were enrolled following the morning milking. Before enrollment, cows were weighed. Following enrollment, cows were moved to an open-sided, freestall barn bedded with sand, along with other postpartum cows. Cows were milked at 0700, 1700, and 2200 h and fed TMR once daily in the morning. The diet was formulated to meet or exceed nutrient requirements for high-producing lactating dairy cattle (NRC, 2001). Cows were provided water ad libitum.

Cattle received oral ASA treatments at enrollment and 24 h later (following the 0700 h milking). A bolus gun was used to administer 3 to 5 boluses of 480 grain (31,104 mg/bolus) ASA (MWI Animal Health) to achieve the target dose of 200 mg/kg of BW. Milk and whole blood were obtained at each time point for SA concentration determination.

Time points for milk collection were before ASA administration (0 h) and 10, 15, 24, 34, 39, 48, 58, 63, 72, 82, 87, 96, 106, 111, and 120 h after ASA administration. Each milk collection time point occurred when cows were scheduled to enter the milking parlor. Cows were milked into floor pails so the entire milking was obtained. Following milking, the milk was well stirred and a milk sample was collected in sample vials (Thermo Fisher Scientific). The milk

samples were immediately placed on ice and transported to the laboratory for storage at −20°C until analysis.

Time points for blood collection were before ASA administration and 2, 6, 10, 24, 34, 48, 58, 72, 82, 96, 106, and 120 h after ASA administration. At each collection time point, cows were restrained in a chute or head-lock, and 6 mL of whole blood was obtained by jugular or coccygeal venipuncture using a 1.5 in 18- or 20-gauge needle. Blood samples were collected using sterile 6-mL lithium heparin-coated tubes (Vacuette, Greiner Bio-One). Blood was immediately placed on ice, transported to the laboratory, and centrifuged (4,000 × g for 10 min at 4°C). Plasma was harvested and stored at −80°C until analysis.

Plasma and milk salicylic acid analysis

Salicylic acid concentrations in milk and plasma samples were determined using ultra performance liquid chromatography triple quadrupole MS (UPLC-MS/MS). Salicylic acid (analytical standard) and salicylic acid-d$_4$ (SA-d$_4$; internal standard, Cerilliant) were purchased from Sigma-Aldrich Inc. Ultrapure water (18.2 MΩ-cm) was obtained from an on-site system (Thermo Fisher Scientific). Optima LC/MS grade acetonitrile, ammonium formate, and ammonium hydroxide, and HPLC grade 85% phosphoric acid were obtained from Thermo Fisher Scientific. The LC/MS and HPLC grade methanol were obtained from Honeywell (Burdick and Jackson). Formic acid (MS standard) was obtained from Waters Corp. Blank filtered lithium heparin bovine plasma (negative control plasma; NCP) was obtained from Lampire Biological Laboratories Inc. Negative control milk was obtained from one of the animals enrolled in the study before ASA administration.

Before sample preparation, plasma samples from 2, 6, 10, and 34 h were diluted 500-fold, and samples from 24 and 48 h were diluted 100-fold with NCP; the remaining plasma samples

were prepared undiluted. The lipid layers from the milk samples in the collection vials were removed, and 1-mL aliquots from each milk sample were centrifuged at 1,500 × g at 4°C for 10 min. After centrifugation, the remaining lipid layers were removed and the milk samples were vortexed.

Salicylic acid stock solution was prepared at 1,000 μg/mL in methanol and stored at −20°C. Salicylic acid-d4 100 μg/mL stock solution was stored in a glass vial (Waters Corp.) at −20°C. A 100 μg/mL working stock solution of SA was prepared daily by diluting the 1,000 μg/mL stock in 4% phosphoric acid. A 0.050 μg/ mL working solution of SA-d4 was prepared daily by diluting the 100 μg/mL stock in 4% phosphoric acid. Working solutions for SA calibration standards (STC) and quality controls (QC) were prepared fresh daily in NCP or negative control milk. The STC were prepared at 8 concentrations (0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.500, and 5.000 μg/mL SA) and QC were prepared at 3 concentrations (0.180, 1.800, and 3.600 μg/ mL SA).

For the STC, QC, negative controls, and internal standard controls, 100 μL of NCP or negative control milk was aliquoted. To this, for the STC and QC, 10 μL of the appropriate STC or QC stock and 190 μL of 0.05 μg/mL SA-d4 in 4% phosphoric acid were added. To the internal standard controls, 10 μL of 4% phosphoric acid and 190 μL of 0.05 μg/mL SA-d4 in 4% phosphoric acid were added. To the negative controls, 200 μL of 4% phosphoric acid was added. For the samples, 100 μL of plasma or milk was combined with 10 μL of 4% phosphoric acid and 190 μL of 0.05 μg/mL SA-d4 in 4% phosphoric acid. Samples, STC, QC, negative controls, and internal standard controls were vortexed and then centrifuged at 1,500 × g at 4°C for 10 min.

Analytes were extracted via solid phase extraction using Oasis MAX 96-well μElution Plates (30μm; Waters Corp.) and a solid phase extraction positive pressure manifold (Positive

Pressure-96 Processor, Waters Corp.). The solid phase extraction cartridges were conditioned with 300 µL methanol followed by 300 µL 18.2 MΩ-cm water. After 300 µL sample, control, STC, or QC solution was loaded into the appropriate wells, the cartridge was washed with 300 µL 5% ammonium hydroxide in 18.2 MΩ-cm water followed by 300 µL methanol. Eluate was collected in a clean collection plate (96-well, 2 mL; Waters Corp.) using 50 µL 2% formic acid in acetonitrile-methanol (60:40) and 50 µL 18.2 MΩ-cm water was added to all collection plate wells. The collection plates were covered with pre-slit silicone cap mats (Waters Corp., Milford, MA) and vortexed gently.

Collection plates were loaded onto an ACQUITY HClass PLUS UPLC system (Waters Corp.). Chromatographic separation was achieved using an ACQUITY UPLC HSS T3 C18 column (100 × 2.1 mm, 1.8 µm; Waters Corp.) kept at 40°C. The UPLC mobile phases consisted of 0.1% formic acid in 5 mM ammonium formate in water (mobile phase A) and 0.1% formic acid in 5 mM ammonium formate in acetonitrile-water (90:10; mobile phase B). A gradient program was used to achieve analyte separation. After sample injection (0 min), a combination of 99% mobile phase A and 1% mobile phase B was linearly changed to a combination of 0% mobile phase A and 100% mobile phase B until 1.49 min. Mobile phase B was linearly reversed to 1% at 2.0 min, and the original mobile phase mixture was held from 2.01 min to 3 min. The flow rate was 0.6 mL/min and the sample injection volume was 2 µL. The LC effluent was diverted to waste for the first 0.7 min and the last 1.0 min of each chromatographic run.

The mass spectrometer was a Xevo TQ-S tandem mass spectrometer (MS/MS) equipped with a Zspray electrospray ionization interface set in negative ion mode (Waters Corp.). Data were acquired and processed by MassLynx and TargetLynx software, respectively (Waters Corp.). The quantifying transition for SA was m/z 137.0636→92.8904 and the qualifying

transition was m/z 137.0636→64.8659. The quantifying transition for SA-d4 was m/z 141.1081→96.9367. The cone voltage and collision energy for the SA quantifying and qualifying transitions were −42 V/−14 V and −42 V/−22 V, respectively. The parameters for SA-d4 were −44 V/−16 V. The dwell time for all compounds was 3 ms.

The concentrations of the eluted standards were 0.0025, 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, and 0.500 µg/mL. The concentrations of the eluted QC were 0.018, 0.180, and 0.360 µg/mL. The limit of detection (LOD) was 0.0025 µg/mL and the lower limit of quantification (LLOQ) was 0.0043 µg/mL. The standard curve was linear from 0.0025 to 0.500 µg/mL; the correlation coefficient was accepted if it was at least 0.975. The intra-day accuracy and precision were determined by analyzing replicates of SA at 3 different QC levels. Inter-day accuracy and precision calculated by analyzing the 3 levels of QC samples were determined to be 108.38 and 4.29%, respectively.

Plasma pharmacokinetic analysis

Pharmacokinetic analysis based on each cow's plasma concentration time curve was performed using PKSolver (Zhang et al., 2010) in Excel (Microsoft Inc.). Using the semi-logarithmic plots of SA, noncompartmental analysis was performed with uniform weighting based on statistical moment theory. Average peak plasma SA concentration (C_{max}) and time to peak concentration (T_{max}) were determined. The log-linear portion of the terminal section of the log plasma concentration time curve was used to calculate the average terminal elimination rate constant for plasma (λ_z) using a linear regression technique. The average terminal half-life of plasma ($T_{1/2\lambda z}$) was determined using the equation $T_{1/2\lambda z} = \ln2/\lambda_z$. The average area under the concentration time curve from 0 to 120 h (AUC0–120h) was calculated using the linear-log trapezoidal method. To account for total drug exposure, the average area under the plasma

concentration time curve and area under the first moment curve were extrapolated from the first measurement to infinite time ($AUC_{0-\infty}$ and $AUMC_{0-\infty}$, respectively). All plasma SA concentrations were above LLOQ. Time versus concentration figures were produced using a commercially available software (GraphPad Prism 9.0).

 Estimation of milk withdrawal interval

A milk WDI for SA was estimated using the US FDA Milk Discard App in R (version 1.1.0, R Studio) adapting methods from Smith et al. (2020) and Meira et al. (Meira et al., 2022). A WDI was calculated for a whole-herd treatment scenario and an individual animal entering the bulk tank. In the whole-herd treatment scenario, the tolerance in the application was set to the LOD of 0.0025 µg/mL, as we observed no established tolerance for SA in milk in the United States. In the second scenario, a tolerance limit of 0.0075 µg/mL was used for the individual animal WDI, where milk enters a commingled bulk tank. This tolerance limit was 3 times the assay LOD and is based on the allowed correction set forth by the FDA (FDA CVM, 2022) which assumes that no more than one-third of the milk in a bulk tank will come from treated cows. To satisfy linearity and homoscedasticity assumptions, certain time points were excluded from the analysis per the US FDA guidelines (Smith et al., 2020). For cows 1, 4, and 9, time points 10, 15, 24, and 34 h after the second ASA dose were used. For cow 2, time points 10, 15, 24, and 39 h after the second ASA dose were used. For cows 3, 7, and 10, time points 10, 15, and 24 h after the second ASA dose were used. For cows 5, 6, and 8, time points 10, 15, and 87 h after the second ASA dose were used.

The application was set to estimate a milk WDI using the methods described in the US FDA Guidance for Industry #3: General Principles for Evaluation the Human Food Safety of New Animal Drugs Used in Food-Producing Animals (FDA CVM, 2022). A 99th percentile

tolerance limit with a 95% confidence was used. The US FDA Milk Discard App requires a minimum of 10 animals with samples analyzed in triplicate. To satisfy this requirement, Monte Carlo simulation was performed in Excel using the mean concentrations and the standard deviations from each time point to generate 2 replicate values. Samples with concentrations above the LOD were used in the model, including 2 samples that had concentrations between the LOD and LLOQ. Six samples had concentrations below the LOD; these were not included in the milk withdrawal analysis or when calculating concentration means and standard deviations for Monte Carlo simulation. The concentrations of these 6 samples were recorded as 0 μg/mL.

Prostaglandin E$_2$ metabolite analysis

Plasma PGEM concentrations were determined using a commercially available competitive ELISA (Prostaglandin E$_2$ Metabolite ELISA Kit; Cayman Chemical). The protocol supplied by the manufacturer was followed except for the following modifications: (1) during sample purification, samples were centrifuged for 5 min at 3,000 × g and 4°C; (2) 300-μL sample volume and corresponding buffer volumes were used for derivatization; (3) the ethyl acetate extraction step was not performed. The inter-assay CV and intra-assay CV were 9.26 and 18.7%, respectively. The average LLOQ was 1.80 pg/mL. Data were analyzed using a commercially available data analysis tool (MyAssays Desktop). Average PGEM concentrations, average percent changes in PGEM concentrations from the baseline, and the associated standard deviations were calculated using Microsoft Excel. The 80% inhibition concentration of plasma SA was determined using a nonlinear regression technique (GraphPad Prism 9.0).

RESULTS

No adverse events were noted in any of the cows throughout the study period.

The log-transformed mean values for milk and plasma SA concentrations versus time are shown in Figure 2.1; plasma SA $T_{1/2\lambda z}$ is reported as the harmonic mean. Pharmacokinetic parameters are summarized in Table 2.1.

Salicylic acid was present in the plasma of all cows at 0 h at an average concentration of 0.1 μg/mL. Following oral administration, plasma SA was present above LLOQ in all cows through 96 h after the last treatment; average SA concentrations were similar to baseline concentrations at 48 h after the last treatment and below baseline concentrations at 72 and 96 h after the last treatment. Secondary SA peaks were observed at 58 and 82 h after the last treatment.

Salicylic acid in the milk of all cows was undetectable at 0 h. Following oral administration, SA was present above the LLOQ in the milk of all cows through 15 h from the last treatment and was undetected by 48 h after the last treatment. A secondary SA peak was observed at 87 h after the last treatment in 3 cows (average concentration across all cows: 0.0019 μg/mL; average concentration for the 3 cows with detectable SA: 0.0063 μg/mL). In one cow, SA was undetected at 34 h after the last treatment but was detected again at low levels at 39 h. The estimated milk WDI using the US FDA application was determined to be 156 h after the last treatment in the whole-herd treatment scenario and 120 h for the second scenario, in which milk from an individually treated cow is diluted in the bulk tank (Figure 2.2).

Plasma PGEM data are summarized in Table 2.2; percent changes in PGEM concentrations are shown in Figure 3. The 80% inhibitory concentration (IC_{80}) for plasma SA was determined to be 67 μg/mL.

DISCUSSION

The main findings of the present study were as follows: (1) plasma SA reached an average peak concentration of 96.6 μg/mL at 2.4 h after ASA administration; (2) milk SA reached an average peak concentration of 0.23 μg/mL; (3) milk withdrawal modeling indicated SA levels were below the tolerance limit by 156 h after final ASA treatment, considering whole-herd treatment, and below the tolerance limit by 120 h after the final ASA treatment, considering individually treated animals and a bulk tank factor adjustment; (4) plasma PGEM concentrations were reduced for up to 12 h after ASA administration; (5) the IC_{80} of SA in plasma was 67 μg/mL.

The US Food and Drug Administration, Center for Veterinary Medicine (2015) recommends that 20 dairy animals be used for milk residue studies for establishing WDI for veterinary drugs approved for use in food-producing animals. However, based on the existing literature describing ASA pharmacokinetics in nonlactating dairy cattle, the widespread use of ASA in the dairy industry and in recent research, and the relative safety of ASA (Damian et al., 1997), we elected to enroll 10 cows.

Salicylic acid has been detected in the plasma of cows who have not been treated with ASA, likely due to consumption of forages, which contain salicylates (Gingerich et al., 1975, Anderson et al., 1979). Gingerich et al. (1975) state that salicyluric acid is normally found in the urine and milk of cows. The plasma SA concentrations we observed before ASA administration were lower than previously reported values (Gingerich et al., 1975), potentially due to differences in diet composition or SA detection methods. Anderson et al. (1979) reported detection of SA before ASA administration but did not indicate the SA concentration. However, visual comparison of the SA concentration graphs suggests that the baseline SA concentrations

detected by Anderson et al. (1979) were lower than those detected by Gingerich et al. (1975). In the study by Gingerich et al. (1975), dietary inclusion of forages with higher salicylate content than the diets in the current study may have been responsible for the higher SA levels detected before ASA administration. Both Gingerich et al. (1975) and Anderson et al. (1979) used a method of extraction and spectrophotometry to quantify plasma salicylate and were able to detect concentrations at approximately 5 µg/mL or greater. Delayed analysis (MacDonald et al., 1965) or the presence of interfering substances in the samples could have altered the values reported by Gingerich et al. (1975), but these possibilities cannot be confirmed. The method employed by Gingerich et al. (1975) and Anderson et al. (1979) was likely much less sensitive than the method implemented in the current study and therefore may not have been able to detect SA at the levels reported in this study. In the present study, ultra performance liquid chromatography triple quadrupole MS (UPLC-MS/MS) was used to quantify plasma SA; the LOD and LLOQ were 0.0025 µg/mL and 0.0043 µg/mL, respectively. Improving assay sensitivity is important for providing more accurate drug pharmacokinetic profiles, which can inform decisions such as drug dosing protocols and withdrawal recommendations.

The secondary peaks observed in milk and plasma may be due to environmental contamination by urine or feces containing SA, consumption of salicylate-containing forages, or both (Gingerich et al., 1975, Anderson et al., 1979, Bates et al., 2020). Considering that the secondary peaks in plasma were consistently observed at the evening milkings—after the morning feeding time—they are perhaps more likely due to forage consumption than environmental contamination and subsequent drug transfer. Even though drug transfer has been reported for other NSAID in swine and horses (Popot et al., 2007, Hairgrove et al., 2019, Bates et al., 2020), acetylsalicylic acid resulting in detectable plasma or milk SA concentrations in

dairy cattle has no drug transfer data. Anderson et al. (1979) did report urine salicylate concentrations in cattle for 84 h following ASA administration, so it is possible that urinary contamination of the environment resulted in the observed rebound SA concentrations. This would help explain the relatively high rebound milk SA levels at 96 h after the last treatment. The smaller secondary peaks we observed in milk at 39 and 87 h after the last treatment are more easily explained by forage consumption. Future studies could be designed to determine the exact reason for these late secondary SA peaks. This information could guide the actions of regulatory officials if low SA residues are detected in milk samples of cows reportedly meeting ASA withdrawal recommendations.

A comparison of plasma salicylate PK profiles in 7 publications is presented in Table 2.3. These previous studies investigated the PK profiles of SS following a variety of treatment regimens and used different quantification methods than in the present study. The 2 earliest publications (Gingerich et al., 1975, Anderson et al., 1979) used extraction and spectrophotometry to determine salicylate concentrations, whereas some later studies have used fluorescence polarization immunoassay (Coetzee et al., 2007, Kotschwar et al., 2009, Baldridge et al., 2011, Bergamasco et al., 2011). These methods of determining SA concentrations have higher LOD than the method described in this study; the results reported by Gingerich et al. (1975) suggest that the extraction protocol had a sensitivity around 5 µg/mL, which was also the LOD of the fluorescence polarization immunoassay used in the later studies. The low LOD of the method used in the present study improves the resolution and utility of the PK data. In the previous studies describing the PK profile of salicylate, the drug administered was often different (SS versus SA), doses administered were smaller (50 or 100 mg/kg of BW), or the doses were administered via a different route (e.g., intravenous, free-choice through the water) than in the

present study. The more recent studies investigating the effects of aspirin (Farney et al., 2013a, Farney et al., 2013b, Carpenter et al., 2016, Barragan et al., 2020a, Barragan et al., 2020b, Barragan et al., 2020c, Barragan et al., 2021) have not described PK profiles of SA.

The greater average C_{max} reported here compared with that reported by Gingerich et al. (1975) may be appropriate, considering the higher target dose used in this study. However, the multiple dose aspirin experiment conducted by Gingerich et al. (1975) had minimal blood sampling times, so the peak concentration values presented in the figures may not be an accurate representation of the true plasma salicylate C_{max}. In a study conducted by Anderson et al. (1979), 3 nonlactating female Holstein cows were administered SA orally at 100 mg/kg of BW at 0, 12, 25, and 36 h; the plasma salicylate C_{max} value was similar to the values reported in this study. Considering the similarity between treatment regimens in the study conducted by Gingerich et al. (1975) and Anderson et al. (1979), it is likely that blood collection in the study by Gingerich et al. (1975) was not properly timed to observe the plasma salicylate peaks, and a lower average C_{max} was thus observed. Baldridge et al. (2011) reported a lower mean C_{max} value than that reported here, which is most likely due to differences in dosage route: free-choice SA was administered in water at a concentration between 2.5 and 5 mg/mL, with doses ranging from 13.62 to 151.99 mg/kg. The higher C_{max} values by Coetzee et al. (2007) (intravenous SS group), Kotschwar et al. (2009), and Bergamasco et al. (2011) are reasonable, considering that SS was administered intravenously. In Holstein calves administered oral aspirin at 50 mg/kg of BW immediately before castration, plasma salicylate levels did not exceed 10 µg/mL (Coetzee et al., 2007). Overall, the differences in C_{max} among the different studies likely reflect the variation in dosing and the timing of sample collection. Although AUC is a better indicator of drug exposure

than C_{max}, it was less frequently reported. Future research should focus on reporting AUC values to more accurately compare drug exposure differences among studies.

The T_{max} reported in this study is similar to the values reported by Gingerich et al. (1975) and Anderson et al. (1979); any numerical differences are likely due to random variation or different sampling times. The T_{max} reported by Baldridge et al. (2011) is significantly longer, which may be explained by the dosage route (free-choice through the water versus via oral bolus in the present study). Because calves were self-medicating, the timing of peak water intake may have artificially increased T_{max}.

A summary by the Center for Veterinary Medical Products (CVMP, 1999) reported an absorption half-life of 2.9 h for cattle administered oral aspirin (20 to 100 mg/kg of BW); in the same report, following intravenous administration of ASA DL-lysine to cattle at 90 mg/kg of BW, the $T_{1/2\lambda z}$ was found to be 36.5 min. Comparing the values in Table 2.3, the $T_{1/2\lambda z}$ reported in the studies evaluating oral administration of ASA or SS are considerably longer than the $T_{1/2\lambda z}$ of salicylate following intravenous administration of SS. The relatively slow absorption of aspirin from the rumen—the absorption half-life is approximately 3 h (Gingerich et al., 1975, Anderson et al., 1979, CVMP, 1999)—is one potential reason for the prolonged $T_{1/2\lambda z}$ in these studies. Even though a flip-flop phenomenon is possible [the rate of absorption, rather than the rate of elimination, is the limiting step in final drug elimination (Toutain and Bousquet-Mélou, 2004b)], this does not fully explain the greatly extended $T_{1/2\lambda z}$ we observed in the present study. We did observe a much higher $T_{1/2\lambda z}$ in the present study than was reported by Gingerich et al. (1975) or Anderson et al. (1979). Because the dose used in the present study was double the dose used in the earlier studies, it is possible that a metabolism pathway was saturated and we thus observed a longer $T_{1/2\lambda z}$. Salicylate and SA are primarily metabolized through glucuronidation

pathways in the liver (CVMP, 1999). However, the authors feel two other explanations for the extended $T_{1/2\lambda z}$ are more robust. First, improved assay sensitivity may have allowed detection of a secondary terminal phase in the present study. Toutain and Bousquet-Mélou (2004b) have shown that terminal half-life is heavily influenced by assay sensitivity; as assay sensitivity improves, terminal half-life will appear to increase due to detection of late terminal phases. Second, decrease in drug clearance due to altered postpartum physiology may have increased $T_{1/2\lambda z}$. In a comparison of the pharmacokinetic profiles of meloxicam in postpartum and mid-lactation cows, Gorden et al. (2018) reported that C_{max}, T_{max}, and mean residence time were increased and clearance was decreased in postpartum cows; the reduction in clearance resulted in an increase in drug exposure and relative bioavailability. In addition, the authors detected higher levels of meloxicam in milk in postpartum cows compared with mid-lactation cows at all time points. However, they did not detect any difference in $T_{1/2\lambda z}$. Furthermore, Warner et al. (2020) reported similar findings in a study evaluating the pharmacokinetics of both intravenous and oral meloxicam in postpartum versus mid-lactation cows. In this study, the authors also reported a significantly longer $T_{1/2\lambda z}$ in the postpartum cows. The authors postulated that these clearance differences could have been due to changes in plasma protein binding or reduced liver enzyme levels in postpartum cows. Considering this evidence for change in NSAID clearance in postpartum cows, it is likely that the postpartum cows used in this study had decreased clearance of SA compared with cows involved in previous studies evaluating PK profiles after oral ASA administration. Because we did not administer ASA intravenously, we were unable to quantify clearance or bioavailability in this study (Toutain and Bousquet-Mélou, 2004a). The bioavailability of oral ASA in dairy cattle has been reported to be 70% (Gingerich et al., 1975). The present study was also not designed to measure changes in plasma protein, protein binding,

or liver enzymes. Further research investigating changes in plasma protein-drug interactions and metabolic enzyme expression in the liver could determine the differences in NSAID pharmacokinetics in postpartum dairy cows compared with cows at other lactation stages. Such research would be useful in optimizing NSAID treatment protocols for cows in different production stages. In summary, increases in assay sensitivity and postpartum decreases in drug clearance in the present study are likely responsible for the longer $T_{1/2\lambda z}$ observed. Future research is needed to compare clearance and bioavailability of SA in postpartum and mid-lactation cows following oral ASA administration.

No data are currently available on SA in the milk of postpartum dairy cattle, so the data reported here are novel. Postpartum cows were specifically selected as the study population based on recent efforts by Barragan et al. (2020a, 2020b, 2020c, 2021) that describe positive benefits of ASA given at 200 mg/kg in the postpartum period. The use of ASA for an anti-inflammatory indication following parturition would constitute an extra-label drug use (ELDU). Under the guidance of the Animal Medicinal Drug Use Clarification Act, the prescribing veterinarian would be responsible for determining an appropriate WDI (FDA, 1996). We determined the C_{max} of SA in milk to be 0.23 µg/mL. Our findings suggest that a milk withhold period of 120 h (5 d) would be most appropriate for cattle in the immediate postpartum period to meet US FDA tolerance guidelines for unapproved drugs. Our description of the PK and milk residue profiles of SA in lactating dairy cows will help producers make informed treatment decisions and ensure food supply safety. Though our data can inform withdrawal decisions, the methods employed in this study are not practical for field use due to their time-intensive nature. To facilitate SA residue detection in milk, future research should focus on developing a rapid assay. Although aspirin is considered to be of low regulatory concern, a 24-h milk WDI is

currently recommended based on potential risk for individuals with Reye's syndrome (Damian et al., 1997). However, the risk of Reye's syndrome is correlated with exposure to high doses of sodium acetylsalicylate, which are unlikely to be obtained through consumption of residues in milk (CVMP, 1999).

When estimating milk WDI, we included all data above the assay LOD. This decision was based on recent publications describing the issues with defining LLOQ and excluding data that fall below that level (Jelliffe et al., 2015, Woodward and Whittem, 2019). In general, assay LLOQ is determined based on an arbitrary threshold for sample variation (CV%; often set at >20%); when measurements lie below this threshold, they are often discarded form the analysis (Jelliffe et al., 2015). However, CV% increases as measurements approach zero, so the establishment of an LLOQ based on this information is erroneous and may exclude valuable data (Jusko, 2012, Jelliffe et al., 2015). Woodward and Whittem (2019) proposed 3 options for handling data below LLOQ: (1) discard data below LLOQ, (2) censor data using maximum likelihood estimates, and (3) use data below LLOQ without adjustment. Keizer et al. (2015) reported improved model performance and precision and decreased bias in population PK analyses when using all data above LOD compared with other methods of censoring data below LLOQ. This effect was more pronounced when the proportion of data below LLOQ was larger. In the present study, only 2 milk samples were below LLOQ and above LOD. Compared with a model that excluded data below LLOQ, our final model that included all data above LOD resulted in a longer bulk tank factor WDI (120 vs. 108 h) and a shorter whole-herd WDI (156 vs. 168 h). Two other possible combinations of time points were able to be modeled, but these models had a poorer fit and were thus not selected over the final model.

Prostaglandin E_2, or PGEM, concentrations in postpartum dairy cattle have been minimally reported in the literature. Farney et al. (2013a) reported an elevation in the total concentration of plasma eicosanoids after cessation of oral treatment with sodium salicylate in drinking water, but they did not provide reports on all individual eicosanoids evaluated. Prostaglandin E_2 as a primary mediatory of inflammation and pain (Myers et al., 2010), is of particular interest in pharmacodynamic analysis. We observed reduction in PGEM concentrations at plasma SA levels of approximately 700 and 425 μM at 2 and 6 h, respectively. Myers et al. (2010) reported that 300 μM of aspirin resulted in reduction in prostaglandin E_2. The discrepancy between our results and those of Myers et al. (2010) can be explained by the weaker inhibition of cyclooxygenase by SS compared with ASA (Mitchell et al., 1993). However, considering the short half-life of ASA in plasma (Gingerich et al., 1975), the active metabolite SA may play a larger role than ASA in inhibiting prostaglandin E_2 production following oral administration of ASA. We observed large standard deviations in both PGEM concentrations and change in PGEM concentrations following ASA administration. Thus, although we did report at least 6 h of prostaglandin inhibition, this result may not be truly representative of the response of postpartum dairy cattle. Wischral et al. (2001) reported that, compared with cows without retained fetal membranes, those with retained fetal membranes had significantly higher PGEM concentrations at 24, 48, 72, and 120 h before and 12 h after calving and lower PGEM concentrations 1 h after calving. Even though none of the cows in this study had retained fetal membranes, it is possible that other, undetected health events could have similarly altered PGEM concentrations, resulting in the large standard deviations observed. It is also unclear whether the transient reduction in prostaglandin production reported here is biologically significant. Future research should focus on establishing reference ranges for PGEM concentrations in postpartum

dairy cattle and determining the long-term effects of ASA on postpartum inflammation and whole-lactation production and health. Research such as that conducted by Vailati Riboni et al. (2015) analyzing adipose and liver gene expression could help elucidate long-term effects of ASA administration and help determine whether the transient inhibition of prostaglandin production reported here is clinically meaningful.

Literature suggests that IC_{80} is more closely correlated with analgesia than half-maximal inhibitory concentration (IC_{50}) (Huntjens et al., 2005); thus, we chose to calculate IC_{80}. The IC_{80} value of 67 μg/mL determined in the present study is more than twice the 30 μg/mL therapeutic level previously reported (Gingerich et al., 1975). Gingerich et al. (1975) stated that they selected the 30 μg/mL therapeutic level based on therapeutic minimum levels in humans, the presumed difficulty of achieving greater levels in cattle, and the alleviation of pain observed in 2 of the animals enrolled in the study. In the previous study (Gingerich et al., 1975), no lactating cows were enrolled and no pharmacodynamic evaluation was performed. Lactating and nonlactating dairy cattle likely exhibit different NSAID PK profiles (Gorden et al., 2018, Warner et al., 2020) and the therapeutic level of SA in lactating dairy cattle may differ from that in other cattle. Our analysis of PGEM concentrations and calculation of IC_{80} is a more objective pharmacodynamic measure than lameness evaluation, which was used by Gingerich et al. (1975). Hence, the therapeutic level we report here may be more accurate and reliable than that previously reported. More comprehensive analyses of the pharmacodynamic effects of NSAID in postpartum dairy cattle are needed to refine treatment recommendations for managing postpartum inflammation.

CONCLUSION

The results of the present study suggest that the current 24-h milk withdrawal recommendation for cattle treated with ASA may require revision to 120 h after ASA treatment.

Furthermore, these data suggest that ASA administration transiently reduces prostaglandin production for up to 12 h. Given that aspirin is not approved for use in lactating dairy cattle in the United States, producers must consult with a veterinarian and demonstrate a valid veterinarian-client-patient relationship before initiating use of ASA in postpartum dairy cattle. Furthermore, because aspirin is not approved by the FDA, ELDU requires adherence with Animal Medicinal Drug Use Clarification Act, which stipulates that ELDU is permitted only if the well-being of the animal is threatened, and that ELDU for production purposes is strictly prohibited. Further research should focus on determining the etiology of the secondary SA peaks following ASA administration and expounding on the pharmacodynamics of ASA and its long-term effects on dairy cow health. Evaluating the differences in NSAID pharmacokinetics between postpartum cows and cows at other production stages and determining reference values for PGEM in postpartum dairy cattle may allow for more robust research and refinement of current NSAID treatment protocols.

Funding and disclaimer

Figure 2.1. Log-transformed average salicylic acid (SA) concentrations in plasma and milk in 10 postpartum Holstein dairy cattle following oral administration of acetylsalicylic acid at 200 mg/kg of BW.

Figure 2.2. Two milk withdrawal interval (WDI) models for salicylic acid (SA) in postpartum dairy cattle after the last of 2 administrations of acetylsalicylic acid at 200 mg/kg of BW, 24 h apart. (A) Whole-herd treatment scenario. (B) Bulk tank dilution scenario.

Figure 2.3. Average percent changes in plasma prostaglandin E_2 metabolite (PGEM) concentrations compared with baseline (0 h) values in 10 postpartum Holstein dairy cattle following the first of 2 oral administrations of acetylsalicylic acid at 200 mg/kg of BW. Error bars represent standard deviations.

Table 2.1. Pharmacokinetic (PK) parameters of salicylic acid (SA) in plasma and milk in 10 postpartum Holstein dairy cattle following 2 oral administrations of acetylsalicylic acid at 200 mg/kg BW.[1]

Item[2]	Plasma	Milk
C_{max} (µg/mL)	96.637 (50.412-139.577)	0.229 (0.158-0.337)
T_{max} (h)	2.4 (2.0-6.0)	
AUC_{0-120} (h x µg/mL)	977.17 (784.56-1,403.51)	
$AUC_{0-\infty}$ (h x µg/mL)	978.38 (786.16-1,403.99)	
AUC % extrapolated	0.13 (0.03-0.25)	
$AUMC_{0-\infty}$ (µg/mL x h^2)	13,211.85 (10,046.87-17,965.85)	
λ_z (h^{-1})	0.061 (0.054-0.071)	
$T_{1/2\lambda z}$ (h)	11.49 (9.70-12.79)	

[1]Acetylsalicylic acid was administered at 0 and 24 h. Plasma and milk were collected through 120 h for PK analysis.
[2]Pharmacokinetic results are presented as the geometric mean (minimum-maximum), except for half-life, which is presented as the harmonic mean (minimum-maximum). C_{max} = maximum plasma concentration; T_{max} = time to C_{max}; AUC_{0-120} = area under the curve for the 120 h after the first treatment; $AUC_{0-\infty}$ = area under the curve extrapolated to infinite time; AUC % = portion of the $AUC_{0-\infty}$ that is extrapolated after the final concentration measurement; $AUMC_{0-\infty}$ = area under the first moment curve extrapolated to infinite time; λ_z = slope of the terminal phase; and $T_{1/2\lambda z}$ = terminal half-life.

Table 2.2. Plasma prostaglandin E_2 metabolite (PGEM) concentrations in 10 postpartum Holstein dairy cattle following 2 oral administrations of acetylsalicylic acid at 200 mg/kg BW.[1]

PGEM concentrations		Change in PGEM concentrations from baseline	
Time	Mean ± SD (pg/mL)	Time	Mean ± SD (%)
0 h	106.11 ± 73.89	0 h	-
2 h	35.33 ± 20.37	2 h	-49.31 ± 54.75
6 h	57.97 ± 32.32	6 h	-18.87 ± 84.48
10 h	84.09 ± 49.99	10 h	13.03 ± 129.55
24 h	82.23 ± 56.55	24 h	12.20 ± 145.86

[1]Acetylsalicylic acid was administered at 0 and 24 h. Plasma was collected through 120 h for PGEM analysis.

Table 2.3. Comparison of the plasma pharmacokinetic (PK) parameters of salicylate between the present study and six previous studies.

	Item[1]			
	C_{max}	T_{max}	$AUC_{0-\infty}$	$T_{1/2\,\lambda z}$
Study	$\mu g/mL$	h	$h \times \mu g/mL$	h
Current study[2]	96.637 (50.412-139.577)	2.4 (2.0-6.0)	978.38 (786.16-1,403.99)	11.49 (9.70-12.79)
Gingerich et al. (1975)[3]	63	2-4	N/A	3.7 ± 0.44
Anderson et al., (1979)[4]	117	2-8	N/A	5.8
Coetzee et al. (2007)[5a]	200-230	N/A	219.30 ± 18.23	0.63 ± 0.04
Coetzee et al. (2007)[5b]	≤ 10	N/A	N/A	N/A
Kotschwar et al. (2009)[6]	226.9 ± 4.9	N/A	201.65 ± 8.50	0.62 ± 0.02
Baldridge et al. (2011)[7]	61.134 ± 10.312	41.7	82.05 ± 14.27	N/A
Bergamasco et al. (2011)[8]	198.720 ± 8.220	N/A	192.73 ± 21.22	0.68 ± 0.08

[1]C_{max} = maximum plasma concentration; T_{max} = time to C_{max}; $AUC_{0-\infty}$ = area under the curve extrapolated to infinite time; $T_{1/2\,\lambda z}$ = terminal half-life; N/A = not applicable.

[2]Aspirin (acetylsalicylic acid) administered at 200 mg/kg BW at 0 and 24 h. Pharmacokinetic results are presented as the geometric mean (minimum-maximum), except for half-life, which is presented as the harmonic mean (minimum-maximum).

[3]C_{max} value is based on results from the multiple dose oral aspirin experiment (100 mg/kg BW every 12 h for 5 days). This value was visually estimated from Figure 5 in the publication. The T_{max} and $T_{1/2}$ values are based on results from the single dose oral aspirin experiment (100 mg/kg BW). Standard deviation is listed with $T_{1/2}$ value. Salicylate concentrations determined using an extraction and spectrophotometry protocol.

[4]Sodium salicylate (SS) administered orally at 100 mg/kg BW at 0, 12, 25, and 36 h. C_{max} value was visually estimated from Figure 1 in the publication. Salicylate concentrations determined using an extraction and spectrophotometry protocol.

[5a]SS administered intravenously at 50 mg/kg BW immediately prior to castration. Results are presented as mean ± SE except C_{max}, which was estimated visually from Figure 1. Salicylate concentrations determined using fluorescence polarization immunoassay.

[5b]Aspirin (acetylsalicylic acid) administered orally at 50 mg/kg BW immediately prior to castration. Results were extracted from the discussion.

[6]SS administered intravenously at 50 mg/kg BW four minutes after lameness induction. Results are presented as mean ± SE. Salicylate concentrations determined using fluorescence polarization immunoassay.

[7]SS administered for 5 days surrounding the time of castration and dehorning via free-choice consumption of water containing SS (SS concentrations in water: 2.5-5 mg/mL). Results are presented as mean ± SEM (SEM not reported for T_{max}). Salicylate concentrations determined using fluorescence polarization immunoassay.

[8]SS administered intravenously at 50 mg/kg BW immediately prior to castration. Results are presented as mean ± SEM. Salicylate concentrations determined using fluorescence polarization immunoassay.

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Chapter 3 - Tissue residue depletion of cannabinoids in cattle administered industrial hemp inflorescence[3]

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ABSTRACT

Despite interest in using industrial hemp (IH) in cattle feed, there are minimal safety data on tissues from exposed cattle. This study sought to describe cannabinoid tissue concentrations, estimate withdrawal times for cattle exposed to IH inflorescence, and generate human exposure estimates for Δ9-tetrahydrocannabinol and cannabidiol. Twenty male Holsteins received IH inflorescence orally at 4.22 mg/kg/d cannabidiolic acid for 14 d. Liver, kidney, muscle, and adipose were collected at 1, 2, 3, 5, and 8 days after IH administration and analyzed for cannabinoids using liquid chromatography mass spectrometry. Withdrawal intervals and target tissues were selected based on the slowest-depleting cannabinoid. Δ9-tetrahydrocannabinol was detected in liver, kidney, and adipose and cannabidiol in all tissues. Withdrawal intervals were generated (up to 154 d), however, slow elimination resulted in a large degree of extrapolation for many cannabinoids. Adipose was selected as the target tissue and cannabidiol the marker residue. Human exposure estimates revealed that newborns may exceed the lowest global threshold of 1 µg/kg Δ9-tetrahydrocannabinol; no other age group reached published toxicity levels for Δ9-tetrahydrocannabinol or cannabidiol. Additional work should determine safe levels of cannabinoid exposure in vulnerable populations (i.e. children and adolescents). Our results will inform future discussions regarding the inclusion of IH in cattle feed.

Key words: cattle, exposure, industrial hemp, inflorescence, tissue residue, withdrawal interval

INTRODUCTION

Subsequent to the legalization of cultivation of industrial hemp [IH; *Cannabis sativa* containing ≤ 0.3% of the psychoactive cannabinoid, Δ9-tetrahydrocannabinol (9-THC)] and its reclassification as a non-controlled substance, interest in IH cultivation and byproduct marketing

has continued to increase (Johnson, 2021). Primary markets for IH products include fiber, seeds and seed products, oils, and cannabinoid extracts (e.g. cannabidiol, or CBD) for medicinal use (Kleinhenz et al., 2020b). The processing of IH for its primary market product leaves behind plant "waste" material. Cattle and other ruminants are designed to digest and transform plant fiber – undigestible by most species – into tissue products (meat and milk) for human consumption.

The plasma pharmacokinetics of cannabinoids in cattle administered a single dose and multiple doses of IH have been published (Kleinhenz et al., 2020a, Kleinhenz et al., 2022). Other studies have evaluated tissue concentrations of cannabinoids in cattle fed IH seed meal products, spent hemp biomass (SHB), and unextracted IH leaves and two studies have evaluated the milk residue profile of cattle fed IH silage and extraction byproduct (Wagner et al., 2022, Addo et al., 2023a, Addo et al., 2023b, Smith et al., 2023, Wang et al., 2023). Currently, there are no publications evaluating tissue residues following IH inflorescence administration. As the flowers and leaves contain the highest concentrations of cannabinoids throughout the plant, this is critical information to guide regulatory decisions regarding use of IH byproducts in livestock feed (Kleinhenz et al., 2020b). While a major goal in using IH as a livestock feed is to improve sustainability of IH production by using plant material remaining following cannabinoid extraction, it is prudent to develop recommendations for animal disposition in a "worst case scenario", with exposure to unextracted products.

The objectives of this study were to 1) describe cannabinoid concentrations in tissues of cattle administered IH inflorescence, 2) estimate withdrawal intervals (WDI) for cattle exposed to IH inflorescence, and 3) calculate human exposure estimates for CBD and 9-THC.

MATERIAL AND METHODS

Study design

Experimental procedures were approved by the Institutional Animal Care and Use
Committee at Kansas State University (IACUC #4326). All study activities were conducted in
conformity to requirements from the United States Department of Agriculture, the State of
Kansas, and Association for Assessment and Accreditation of Laboratory Animal Care according
to *The Guide for the Care and Use of Agricultural Animals in Research and Teaching (ADSA
ASAS PSA, 2020)*. Industrial hemp was grown and handled in keeping with licensing
requirements under the Kansas Department of Agriculture Industrial Hemp Research Program
(license numbers: KDA-0621466839 and KDA-0302873296). The cultivar used was Otto II
Stout, which was grown and harvested by K-State faculty at the John C. Pair Horticultural Center
near Wichita, KS during the fall of 2019.

This was a residue depletion study with all animals receiving the same treatment, so no
random allocation was required. Sample size was chosen based on FDA guidance
recommendations of 4 animals per slaughter timepoint (FDA CVM, 2015). The experimental
unit was the individual animal. The study was completed in accordance with ARRIVE
guidelines.

Subjects and housing

Twenty male Holstein cattle (n = 16 bulls, n = 4 steers), approximately 5-7 mo and
weighing (± SD) 294 ± 28 kg were enrolled in the study. Animals were sourced from another
investigator at Kansas State University (IACUC #4225); the animals were initially procured from
a commercial calf raising operation in South Dakota. Animals were enrolled if they were
considered healthy on examination by a veterinarian. Animals had been previously acclimated to

the research facility and were group-housed in outdoor dirt pens with access to shelter throughout the study period. The pen area and shelter space supplied per calf exceeded the guidelines established in the Guide for the Care and Use of Agricultural Animals in Research and Teaching. Animals were fed a custom grain mix twice daily and had ad libitum access to grass hay and water via an automated watering device during the entire study. None of the animals had received IH previously.

Industrial hemp dosing and cattle sampling

Prior to study initiation, the cannabidiolic acid (CBDA) content as a percent of total IH weight was determined using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS/MS) and was used to calculate IH doses on an as-fed basis. (A separate full analysis of the cannabinoid content of the IH cultivar used was performed prior to animal dosing at the Kansas State University Olathe Campus Postharvest Physiology Lab; DEA Registration Number RK0682256.) Cattle received IH at a target dose of 5 mg/kg CBDA by oral bolus every 24 hours for 14 d in order to achieve steady state. Hemp flower material was finely chopped using a food chopper. Ground hemp material was placed in gelatin capsules and the weight of the capsules (with empty capsule weight tared) was recorded. Each capsule contained approximately 4-8 g of ground IH, depending on animal weight, and each animal received 2 capsules per day. Supplementary Table S9 includes dosing information for each animal. Animals were monitored after IH administration to ensure capsules were consumed (not spit out).

Animals were randomly assigned to groups for euthanasia on days 15, 16, 17, 19, and 22 (1, 2, 3, 5, and 8 days after last IH administration). Euthanasia was performed in accordance with AVMA guidelines for euthanasia via penetrating captive bolt and exsanguination. Per humane slaughter guidelines for exsanguination, an incision was made in the ventral neck which included

the jugular veins and carotid arteries. Liver, kidney, muscle, and adipose tissues were collected

from all euthanized animals. Muscle was collected from the semimembranosus and adipose was

collected from the perirenal fat pad.

Tissue cannabinoid analysis

All solvents and chemicals were LC-MS grade and purchased from Fisher Scientific

(Hampton, NH). Ultrapure 18Ω water was obtained in-house with a Millipore Synergy UV-R

system (Burlington, MA). Cannabinoids standards purchased in individual solutions in methanol

or acetonitrile from Cerilliant Corporation (Round Rock, TX) and Cayman Chemicals (Ann

Harbor, MI). Cannabinoids included in the panel were: (+)-11-nor-9-carboxy-Δ9-

tetrahydrocannabinol glucuronide (THC-acid-glu), (−)-11-nor-9-carboxy-Δ9-

tetrahydrocannabinol (THC-acid), cannabicyclolic acid (CBLA), (−)-7-nor-7-carboxy

cannabidiol (CBD-7-acid), (±)-11-hydroxy-Δ9-tetrahydrocannabinol (THC-11-OH),

cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), cannabidiol (CBD), cannabidiolic

acid (CBDA), Δ9-tetrahydrocannabinolic acid A (THCA), cannabigerolic acid (CBGA),

cannabigerol (CBG), Δ9-tetrahydrocannabinol (9-THC), Δ8-tetrahydrocannabinol (8-THC),

cannabichromene (CBC), Δ9-tetrahydrocannabivarin (THCV), cannabichromenic acid (CBCA),

cannabinol (CBN), and cannabicyclol (CBL). Δ9-tetrahydrocannabinol glucuronide was

purchase from ElSohly Laboratories, Inc (Oxford, MS). The deuterated analogs used as internal

standards were: (±)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol-d$_9$ (THC-acid-d$_9$) (±)-11-

hydroxy-Δ9-tetrahydrocannabinol-d$_3$ (THC-11-OH-d$_3$), cannabidiolic acid-d$_3$ (CBDA-d$_3$) and

Δ9-tetrahydrocannabinolic acid A-d$_3$ (THCA-d$_3$), cannabidiol-d$_3$ (CBD-d$_3$), Δ9-

tetrahydrocannabinol-d3 (9-THC-d$_3$), and cannabichromene-d$_9$ (CBC-d$_9$).

Briefly, tissue samples were spiked with a mixture of internal standards and homogenized with a single-probe homogenizer in a mixture of acetonitrile and formic acid 5% in a ratio of tissue to extraction volume of 1:10 (w/v). All extracts were subsequently cleaned-up by dispersive solid phase extraction (dSPE) using Bond-Elut EMR (Agilent Technologies, Santa Clara, CA) followed by QuEChERS with Bond Elut EMR-Lipid Polish (Agilent Technologies, Santa Clara, CA). Calibration standards for muscle, kidney and liver were prepared by spiking negative control tissues to obtain a final concentration of cannabinoids of 1.25, 2.5, 6.25, 12.5, 25, 62.5, 125 ng/g. Quality controls were prepared by spiking negative control tissues with 2, 10 and 100 ng/g of cannabinoids. Cleaned tissue extracts (2.5 mL) were evaporated under vacuum at 40°C. Muscle and kidney residues were reconstituted with 0.25 mL of a mixture of acetonitrile-water (1:1) containing 0.1% formic acid, centrifuged, and transferred to an autosampler vial for LC-MS analysis.

To the liver residues, 2mL of water was added before further clean-up by solid-phase extraction (SPE) using Oasis HLB PRIME SPE cartridges, 6 cc, 200 mg (Waters Co., Milford MA). After loading the extract, the cartridge was washed twice with 2 mL of aqueous methanol 25%, let dry for 1 min and cannabinoids were eluted with 2 x 2 mL of acetonitrile-methanol (90:10). The solvent was evaporated with a vacuum concentrator at 40°C and the residue reconstituted with 0.2 mL of acetonitrile-water (1:1) containing 0.1% formic acid, centrifuged and transferred to an autosampler vial for LC-MS analysis.

For fat tissue samples, matrix-matched calibration standards were prepared by adding 10 µL of cannabinoids stock solutions in acetonitrile (10, 25, 50, 100, 250, 500, 1000 ng/mL) to 80 µL of the negative control fat extract and 10 µL of internal standard solution. Quality controls were prepared at 56.25 ng/g, 112.5 ng/g, 225 ng/g, and 450 ng/g.

Cannabinoids analysis was performed using an Acquity H UPLC and a TQ-S triple quadrupole mass spectrometer (Waters Corp., Milford, MA). The chromatographic separation was performed with a UPLC column Eclipse Plus C18 100 x 2.1 mm, 1.8 μ (Agilent Technologies, Santa Clara, CA) heated at 55°C. The flow rate was set at 0.5 mL/min. The mobile phase consisted of a gradient of water containing 0.1% formic acid (A) and acetonitrile (B) as follows: 0 min: 60% B, 6.50 min: 86% B, 7.50 min-9 min: 100% B, 9.01 min-12 min: 60% B. The total run time was 12 min. The injection volume was 5 μL. The capillary voltage was 3.0 KV, the source temperature 150°C, the desolvation temperature 500°C, the desolvation nitrogen flow at 1000 L/h, and the cone nitrogen flow at 150 L/h. The data acquisition was performed by electrospray ionization (ESI) in positive and negative mode using multiple reaction monitoring (MRM). Data processing was performed using linear regression with a weighing factor of 1/X and was accepted if the coefficient of correlation R^2 was >0.99. Limit of detection, lower limit of quantification, and upper limit of quantification are summarized in Table 3.4. The complete cannabinoid concentration dataset is available in Supplementary Tables S1-S8.

Tissue withdrawal period estimation

Withdrawal estimates were made using the US FDA WithdrawalApp in R Studio. At least two data values at three or more timepoints were required to estimate a withdrawal interval; thus, a withdrawal interval could not be estimated for all cannabinoids detected in each tissue. Not all data points were included for each cannabinoid WDI model in each tissue, in order to satisfy linearity and homoscedasticity of the model. A summary of tissue cannabinoid concentrations and the data points used for each cannabinoid WDI model are available in Supplementary Reports R1-R24. Per GFI guidelines, A 99th percentile upper tolerance limit and a 95% confidence interval were used (FDA CVM, 2022). Based on information from discussions

with the Hemp Feed Coalition, a tolerance of 10 ng/g was used (M. D. Kleinhenz, personal communication, 31 May 2022). Withdrawal intervals were rounded up to the next full day. The tissue-specific WDI and the overall WDI were determined based on the slowest-depleting cannabinoid for individual tissues and across all tissues, respectively. Total CBD was calculated by adding the CBD concentration and (0.877*CBDA) for that sample. Total THC was calculated by adding the 9-THC concentration and (0.877*THCA) for that sample. Total cannabinoid concentrations were calculated by adding all cannabinoids detected in that sample. For individual cannabinoids, all values above LOD were included in the data file loaded into the WithdrawalApp. For calculated values (total CBD, total THC, and total cannabinoids), all values were included.

For liver tissue, withdrawal periods were estimated for CBD, CBDA, total CBD, CBD-7-acid, THCA, total THC, THC-glu, and total cannabinoids. For kidney tissue, withdrawal periods were estimated for CBD, total CBD, THCA, total THC, and total cannabinoids. For muscle tissue, withdrawal periods were estimated for CBDA, total CBD, CBD-7-acid, THC-acid, and total cannabinoids. For adipose tissue, withdrawal periods were estimated for CBD, total CBD, CBD-7-acid, 9-THC, total THC, and total cannabinoids.

Human exposure estimates

Estimates of human exposure to 9-THC and CBD were calculated similarly to a previous report on cannabinoid residues in cattle following administration of hempseed cake (Smith et al., 2023). Exposure estimates were separated by age group and sex, using age groups and fat intake data from Table 11-38 of the Environmental Protection Agency (EPA) Exposure Factor Handbook (EPA, 2018). This table included fat intake data from the top decile of animal fat consumers; data from the 95th percentile of consumers within that decile were used, assuming

that 100% of fat intake was from adipose tissue from cattle administered oral IH inflorescence. For ages 71-81 y and 81+ y, there were not separate data for male and female consumers. Bodyweight (BW) data required for calculation of cannabinoid intake and exposure limits were extracted from the Centers of Disease Control (CDC) anthropometric data from 2015-2018 (for ages 3 y and older) and from the 2000 growth chart publication (for newborn through 2 y) (Kuczmarski et al., 2002, Fryar et al., 2021). When the age ranges from the EPA and CDC publications did not match, the mean BW from the youngest age for the corresponding fat intake group was used (e.g. for fat intake for children aged 2-3 y, the mean 2 y BW was used). For ages 21 y and older, the age ranges of the EPA and CDC publications did not match; the mean BW for the group that was most similar to the fat intake group was used (e.g. for 31-41 y fat intake, mean 30-39 y BW was used). The 1 μg/kg acute reference dose (ARfD) for 9-THC published by the European Food Safety Authority (EFSA) and the 0.15 mg/kg/d (150 μg/kg/d) acceptable daily intake (ADI) for CBD published by the Food Standards Agency were used to calculate allowable exposure limits (EFSA, 2015, Choudhry and Haynes, 2024).

$$(kg\ BW) \times \left(\frac{1\ \mu g\ 9\text{-}THC}{kg}\right)$$

$$(kg\ BW) \times \left(\frac{0.15\ mg\ CBD}{kg}\right)$$

The ARfD represents the threshold of acute toxicity; the value published by the EFSA is the lowest global threshold for 9-THC exposure (Beitzke and Pate, 2021, Smith et al., 2023). Intake of 9-THC or CBD through ingestion of adipose tissue from cattle exposed to IH inflorescence was calculated by the following equation,

$$\left(\frac{ng\ 9\text{-}THC\ or\ CBD}{g\ fat}\right) \times \left(\frac{g\ fat\ intake}{d}\right)$$

where the concentration of 9-THC or CBD represents the 99$^{%}$ CI for concentration on the sampling day with the highest mean concentration (day 2 for both 9-THC and CBD, with concentrations of 74.7 and 371 ng/g, respectively). Finally, the percentages of the allowable exposure limit for 9-THC and CBD represented by the intake values were calculated by:

$$\left[(ng\ 9\text{-}THC\ intake) \times \left(\frac{\mu g}{1{,}000\ ng}\right)\right] \div (\mu g\ 9\text{-}THC\ allowable\ exposure) \times 100\%$$

$$\left[(ng\ CBD\ intake) \times \left(\frac{mg}{1{,}000{,}000\ ng}\right)\right] \div (mg\ CBD\ allowable\ exposure) \times 100\%$$

These estimate procedures for fat, 9-THC, and CBD intake, BW, 9-THC ARfD, and CBD ADI enabled generation of conservative estimates of 9-THC and CBD exposure. While Smith et al. (2023) calculated values for the 10th, 50th, and 90th percentiles of fat intake, only the 95th percentile of the top decile of animal fat consumers was used in the present study to demonstrate the scenario with the highest consumer risk.

RESULTS

No adverse effects were observed in any cattle. Cannabinoid content of IH and average cannabinoid doses are displayed in Table 3.1. The average administered doses of CBDA and 9-THC were 4.22 mg/kg/d and 0.04 mg/kg/d, respectively.

Out of 20 cannabinoids tested (see Supplementary Tables S1-S8), nine were detected in liver: Δ9-tetrahydrocannabinol (9-THC), Δ9-tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabidivarinic acid (CBDVA), cannabicyclolic acid (CBLA), (−)-7-nor-7-carboxy cannabidiol (CBD-7-acid), (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide (THC-acid-glu), and Δ9-tetrahydrocannabinol glucuronide (THC-glu). Six cannabinoids were detected in kidney: Δ8-tetrahydrocannabinol (8-THC), 9-THC, THCA, CBD, CBDA, and (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-acid).

Four cannabinoids were detected in muscle: CBD, CBDA, CBD-7-acid, and THC-acid. Four cannabinoids were detected in adipose: 9-THC, CBD, CBDA, and CBD-7-acid. The psychoactive cannabinoid 9-THC was found in liver, kidney, and adipose. The bioactive cannabinoid CBD and its precursor, CBDA, were detected in all tissues. Average cannabinoid tissue concentrations are shown in Table 3.2. Withdrawal intervals (WDI) are displayed in Fig. 3.1 and Table 3.3. Limits of detection (LOD) and quantification are presented in Table 3.4. Adipose was selected as the target tissue and CBD the marker residue based on the slowest depletion rate (154 days).

Human exposure estimates are presented in Tables 3.5 and 3.6. Newborns exceeded the European Food Safety Authority's 1 µg/kg acute reference dose (ARfD) for 9-THC (males: 173.1%, females: 156.7%). No other group reached this threshold, and no group approached the Food Standards Agency's 150 µg/kg/d (0.15 mg/kg/d) acceptable daily intake (ADI) for CBD (EFSA, 2015, Choudhry and Haynes, 2024). The group with the highest percentage of CBD exposure was newborn males, at 5.7% ADI.

DISCUSSION

This is the first report of cannabinoid tissue residue profiles in cattle administered IH inflorescence. Our target was 5 mg/kg CBDA, based on the 5 mg/kg CBD dose reported for human patients suffering from seizures due to Lennox-Gastaut and Dravet syndromes; this was the target dose for our previous studies (Kleinhenz et al., 2020a, Kleinhenz et al., 2022). The average CBDA and 9-THC doses administered in this study were 4.22 and 0.04 mg/kg/d, respectively.

We detected a total of 11 different cannabinoids, with the widest range found in liver and the slowest depletion in adipose, with an estimated WDI of 154 days for CBD. Thus, adipose

tissue was selected as the target tissue and CBD the marker residue. The psychoactive

cannabinoid 9-THC was detected in liver, kidney, and adipose tissue, but had a shorter WDI than

CBD. The bioactive cannabinoid CBD and its precursor, CBDA, were detected in all tissues.

This was expected, as CBDA was the cannabinoid with the highest concentration in the IH

material. No group reached the 150 µg/kg ADI for CBD. Based on our exposure estimates, only

newborn males and females exceeded the 1 µg/kg 9-THC ARfD.

Many acidic cannabinoids were detected in liver and muscle. Similarly, previous studies

using IH inflorescence, SHB, and IH leaves have consistently reported predominantly acidic

cannabinoids in plasma (Kleinhenz et al., 2020a, Kleinhenz et al., 2022, Fruge et al., 2025,

Irawan et al., 2025). In the current study, we detected THC-acid-glu, THC-glu, 8-THC, 9-THC,

and THC-acid, which were not detected in plasma in our previous studies (Kleinhenz et al.,

2020a, Kleinhenz et al., 2022). The difference in cannabinoid profile of plasma compared to

tissue suggests that there is conversion in the rumen with subsequent distribution to non-central

compartments, tissue-specific metabolism resulting in unique tissue cannabinoid signatures, or a

combination of both. Cultivar differences across studies may contribute to the variation in

cannabinoid profile, but we believe it is unlikely to be responsible for the dramatically increased

number of cannabinoids detected in tissues from this study. The spectrum of cannabinoids

detected in liver is consistent with its role in cannabinoid metabolism reported for other species.

In liver, the cytochrome P450 (CYP) pathway and additional phase II metabolic reactions,

predominantly glucuronidation, occur (Harvey and Brown, 1991, Yamamoto et al., 1995, Jiang

et al., 2011, Gaston and Friedman, 2017). Significant first-pass metabolism has been described in

humans; presumably, this occurs in cattle, which would prevent some cannabinoids from

entering systemic circulation and result in a restricted plasma cannabinoid signature, as we have

reported (Grotenhermen, 2003, Kleinhenz et al., 2020a, Kleinhenz et al., 2022). Rumen and tissue enzymes responsible for cannabinoid metabolism and the extent of first-pass metabolism have not been described in cattle. Similar to our past studies in plasma, we did not detect THC-11-OH—the bioactive and psychoactive metabolite of 9-THC—in tissue (Kleinhenz et al., 2020a, Kleinhenz et al., 2022). While the activity of many cannabinoids is unknown in humans or cattle, identifying pharmacokinetic trends can increase our understanding of metabolic pathways.

Three previous studies have evaluated tissue residues in cattle exposed to hemp products. In a study by Smith et al. (2023) investigating tissue residue profiles of cattle fed hempseed cake, the authors detected no cannabinoids in muscle and observed varying depletion times and concentrations in liver, kidney and adipose. The liver and kidney profiles from that study were similar to each other, with detectable concentrations of various acidic cannabinoids. While no cannabinoids were above the LOD in liver on day 4 (LOD ≤ 2.6 ng/g for all cannabinoids), CBNA and CBDA/THCA were still detectable in kidney on day 8 in 2 of the 4 sampled animals. In adipose, CBD/9-THC, CBDA/THCA, and CBCA were detected on day 0; only CBD/9-THC was detectable on days 1, 4, and 8 of withdrawal. The CBD/9-THC concentration was 10.1 ng/g on day 0 and 6.3 ng/g on day 8.

Compared to the aforementioned study, we detected higher adipose CBD and 9-THC concentrations but similar depletion rates. The previous study utilized an analytical method that could not differentiate isomeric compounds (CBD vs. 9-THC, CBDA vs. THCA), unlike our method. We detected concentrations of CBD and 9-THC at an average of 224 and 47 ng/g, respectively, on day 1 and 143 and 28 ng/g, respectively, on day 8. These concentrations are approximately 4-22X higher compared to the study by Smith et al. (2023). This is expected,

since inflorescence contains more cannabinoids than seeds (Kleinhenz et al., 2020b). Comparing the change in adipose CBD and 9-THC concentrations from day 1 to day 8 in our study versus the study by Smith et al., CBD/9-THC concentrations decrease by about 40% for both studies. This suggests that the elimination rate for both studies during this sampling period was similar. In humans and rats, a depot effect in adipose has been observed: cannabinoids become concentrated and are slowly released over time, resulting in prolonged residues (Johansson et al., 1989, Gunasekaran et al., 2009). This raises concern for a similar phenomenon in cattle, wherein 9-THC or CBD remain in adipose and redistribute to other organs for an extended period due to a slower terminal elimination phase. Because neither study sampled cattle longer than 8 days, we cannot deny the possibility of a slower terminal elimination half-life for the cannabinoids we measured. Long-term elimination data is also lacking for plasma, as we only sampled through 96 h in our single-dose trial and 108 h in our multi-dose trial (Kleinhenz et al., 2020a, Kleinhenz et al., 2022).

A study by Irawan et al. (2025) investigating the depletion of cannabinoids in tissue and milk from cows fed SHB sheds some light on long-term cannabinoid disposition. In that study, Jersey cows were fed 7.5% SHB for 28 days followed by a 28 d withdrawal and a routine dry-off period. There was minimal accumulation of cannabinoids in muscle and the highest concentration in liver. Similar to the present study, the proportions of CBD and 9-THC in adipose were greater than in SHB. The doses in the study by Irawan et al. were approximately 20-fold greater for 9-THC (0.81 vs. 0.04 mg/kg) and approximately 36-fold greater for CBD (15.6 vs. 0.43 mg/kg) than in our study. Based on visual inspection of the data from the prior study, 9-THC and CBD concentrations in adipose on the final day of SHB feeding (similar to our day 1 sample) were approximately 400 and 3,000 ng/g, respectively. This is roughly 8.5-fold

greater for 9-THC and 13-fold greater for CBD than our values. This increase in concentration is not as great as the increased dose between studies. The discrepancy in tissue concentrations could be due to altered accumulation based on body fat percentage and other physiologic differences in the study animals. We used Holstein steers for this study, which presumably had higher bodyfat and metabolic differences compared to the lactating Jersey cows used by Irawan et al. (2025). Even after a 28-day withdrawal from SHB, Irawan et al. reported higher average adipose CBD and 9-THC concentrations (628 ± 828 ng/g and 92 ± 107 ng/g, respectively). While this could be due solely to the increased dose used by Irawan et al.(2025), their observations in milk support the depot theory discussed earlier. Irawan et al. reported higher CBD and CBDA concentrations in milk at 7 d postpartum (90 d after withdrawal) compared to 28 d after withdrawal. Fatty acids are released during the energy-intensive postpartum period, supporting the idea that cannabinoids are stored and later released from adipose into other compartments. Thus, the concentrations of CBD and 9-THC that Irawan et al. reported at the end of the 28-d withdrawal period could simply be due to a slow terminal elimination rate in adipose.

Fruge et al. (2025) reported similar findings in their study evaluating cannabinoid depletion in Holstein steers fed IH leaves. In adipose, THCA was not detected and CBDA was only detected on day 1 of withdrawal. The highest concentrations of CBD and 9-THC in adipose were detected on day 1 of withdrawal (246.6 ng/g and 14.9 ng/g, respectively) with decreased, but detectable, concentrations at 6 weeks (42.7 ng/g and 3.4 ng/g, respectively). Fruge et al. (2025) administered IH leaves at a dose of 3.9 mg/kg CBDA once daily for 14 d, which is similar to our dose. While the day 1 CBD concentration reported by Fruge et al. (2025) is similar to the present study, the 9-THC concentration was much less than the present study. Fruge et al. did not report individual cannabinoid concentrations in the IH they used, but it is likely that cultivar

differences are responsible for the disparity in 9-THC concentrations. Those authors noted large standard deviations in fecal cannabinoid concentrations between animals, suggesting individual variability in digestive and absorptive ability for cannabinoids (Fruge et al., 2025). The inconsistency between individuals, cultivars, and IH forms complicates the ability to interpret and apply depletion data for exposed cattle.

Based on the results from this study, WDI for multiple cannabinoids were modeled. The cannabinoid with the longest WDI was CBD in adipose tissue, making CBD and adipose the ideal marker residue and target tissue, respectively. We expected to see a progressive decline in concentrations over time but observed the highest concentrations of cannabinoids in adipose on day 2. This can be explained by higher average doses received by cattle euthanized on day 2 versus day 1. This aberrant temporal trend may have skewed our WDI models towards overestimation, since the model relies on linear depletion kinetics. Liver had the second longest WDI: 68 d for CBD-7-acid. Our study and prior literature agree that adipose is the tissue with the slowest depletion (Smith et al., 2023, Fruge et al., 2025, Irawan et al., 2025). This is consistent with literature in humans and rats, which has shown persistence of 9-THC in adipose for several weeks (Johansson et al., 1989, Gunasekaran et al., 2009). While improved detection limits could identify a more slowly depleting cannabinoid, data from the present study, prior literature, and communication from regulatory agencies support that CBD and 9-THC are currently the most important cannabinoids. For a majority of cannabinoids in liver, kidney, and muscle, there was little extrapolation from the last data collection point until the estimated WDI. However, for all cannabinoids in adipose and additionally for CBD-7-acid in liver and muscle, tissue concentrations did not appear to have reached a terminal elimination phase by the final collection point. Thus, there was a very large degree of extrapolation in the models, making application of

these WDI values invalid. Nonetheless, the prolonged WDI for cannabinoids in adipose is consistent with prior literature and is useful for confirming adipose as the target tissue for IH exposure.

Compared to prior IH literature, we observed different cannabinoid profiles that could be due to variations in the form of IH administered. In the present study, we did not detect CBCA or CBGA in any tissues, unlike the report by Smith et al. (2023), Irawan et al. (2025) detected CBCA in liver and muscle at the end of the SHB feeding period. Similar to Smith et al., we detected CBDVA in liver, but WDI model assumptions weren't satisfied for the present study. In a study by Addo et al. (2023b) investigating hempseed meal in non-lactating dairy cows, the authors detected no cannabinoids in plasma, rumen fluid, urine, kidney, liver, muscle, or adipose tissues, but did detect CBD and CBDA in the feces of cows receiving either a mix of canola and hempseed meal or hempseed meal alone. Addo et al. (2023b) reported higher CBD intake (63 µg/kg CBD) compared to the Smith study and used an LCMS method with a higher LOD [10 ng/g vs. 0.1 to 3.5 ng/g in the study by Smith et al. (2023)]. Smith reported cannabinoid concentrations in liver and adipose exceeding 10 ng/g. Thus, we are unsure of the reason behind the negative cannabinoid finding in the Addo study. At least some of the difference in cannabinoid profiles between the present study and the studies involving hempseed, leaves, and SHB is likely due to differences in cannabinoid composition and other dietary factors influencing absorption (e.g. pH, protein binding in the feed matrix, heat treatment or other processing, hempseed cake vs. meal, etc.) (Addo et al., 2023b, Smith et al., 2023, Fruge et al., 2025, Irawan et al., 2025). A study evaluating cannabinoid composition in ensiled hemp showed increases in neutral and decreases in acidic cannabinoids over time (Klevenhusen et al., 2024). Comparative cannabinoid pharmacokinetics across IH forms (e.g. inflorescence, leaves, hempseed products,

SHB, silage) and cultivars should be investigated and would be crucial for establishing accurate WDI and feeding recommendations for regulatory agencies and producers.

In addition to the effect of IH form, variable inter-species cannabinoid profiles warrant investigation. Irawan et al. (2024a) fed lambs a control diet or 10% or 20% SHB for 4 and 8 weeks and measured tissue concentrations after a 4 or 0-week withdrawal following final exposure, respectively. They detected 9-THC in the 0 week samples of muscle from lambs fed both levels of SHB but did not detect it after a 4-week withdrawal. The authors reported measurable adipose concentrations of 9-THC, CBD, CBC, and THCA in lambs sampled at 0 weeks. The concentrations of 9-THC in adipose (1.09 μg/g in the low and 4.32 μg/g in the high hemp group) were higher than in muscle (0.16 μg/g in the low and 0.63 μg/g in the high hemp group). Only THCA was detected in adipose after a 4-week withdrawal. Compared to SHB, 9-THC proportions were markedly increased in adipose (21X) and muscle (27X), while CBDA proportions were decreased by about 10-fold in adipose and muscle. We similarly found high concentrations of 9-THC in adipose compared to other tissues but also found high concentrations of CBD and CBDA; we did not detect 9-THC in muscle. In another study, Krebs et al. (2021) fed sheep a control diet or 2 levels of hemp stubble (28% or 56%) for 56 days and euthanized the last day of feeding. The authors detected THCA in the livers of 2 of the 5 sheep in both hemp groups and CBD in the liver of one sheep in the 56% hemp group. In adipose, 9-THC was detected at higher concentrations in the 28% hemp group than the 56% hemp group; THCA was detected in the subcutaneous fat of one sheep in the 28% hemp group. The authors detected 9-THC in muscle from 4 out of 5 sheep in the 28% group, but in only 1 sheep from the 56% group. The reason for the higher 9-THC concentrations in the low hemp group is unknown, especially since dry matter intake was equivalent between groups and digestibility was higher for the high hemp

group. Stevens et al. (2022) fed sheep green hemp biomass for 24 days and collected subcutaneous fat. They detected 9-THC in 4 out of 6 sheep on the final day of feeding (22 d), in all 6 sheep 35 days after last feeding, and in 1 sheep 140 days after last feeding. When investigating the effects of hemp forage in goats, Ran et al. (2024) found no cannabinoids in muscle after the 70-day feeding period.

Comparing the literature in sheep and cattle, the detection of 9-THC in adipose is consistent between species. As opposed to cattle, 9-THC is also reliably detectable in muscle of exposed sheep (Krebs et al., 2021, Stevens et al., 2022, Smith et al., 2023, Irawan et al., 2024a, Irawan et al., 2025). The higher concentrations reported in the studies by Krebs and Stevens (low to mid-hundred μg/kg) are likely due in part to the expression in terms of dry matter (Krebs et al., 2021, Stevens et al., 2022). Without knowing precise tissue water content, it is hard to compare those data to information in cattle or Irawan's study in lambs. However, we can directly compare the concentrations found in the cattle studies to Irawan's findings. The lambs in the Irawan study received 9-THC at 0.7 to 2.6 mg/kg/d, which is 1- to 3-fold higher than their study in cattle (0.81 mg/kg/d), 17- to 65-fold higher than the present study (0.04 mg/kg/d), and 113- to 419-fold higher than the Smith study (average intake of 6.2 μg/kg/d CBD/9-THC) (Smith et al., 2023, Irawan et al., 2024a, Irawan et al., 2025). Adipose concentrations of 9-THC in the lambs from Irawan's study ranged from 1,000 to 4,000 ng/g (low and high hemp groups, respectively; no withdrawal), compared to an average of 500 ng/g (visual estimation) at the end of the intervention period in their study in cattle, 47 ng/g in cattle on sampling day 1 of this study, and 10 ng/g in cattle on sampling day 0 of the Smith study (Smith et al., 2023, Irawan et al., 2024a, Irawan et al., 2025). These values correlate with the differences in cannabinoid intake, suggesting that bioavailability of cannabinoids may be similar in lambs and cattle. However,

three of the compared studies used different forms of hemp. Comparing cannabinoid pharmacokinetics and residues in cattle and lambs fed the same hemp form would be enlightening and remove potential confounding by dietary form.

We performed estimates for human exposure to 9-THC and CBD and used assumptions that visualized the "worst-case scenario" (overestimate exposure). In the present study, newborn males and females exceeded the 1 µg/kg ARfD for 9-THC; no group reached the 150 µg/kg/d ADI for CBD. In the report by Smith et al. (2023), no group evaluated reached the ARfD for 9-THC. As in previous studies evaluating tissues and milk, we did not consider THCA or CBDA, the acidic precursors of 9-THC and CBD, due to regulatory agencies' focus on 9-THC and CBD (Wagner et al., 2022, Smith et al., 2023). Although other studies have evaluated various formulations of IH, inflorescence and leaves contain the highest concentrations of cannabinoids (Kleinhenz et al., 2020b). It is possible that feeding IH inflorescence or unextracted whole-plant material at a higher level than the current study could increase tissue concentrations and prolong WDI. Based on our calculations, adipose concentrations of 9-THC and CBD could be as high as 43 ng/g and 6,481 ng/g, respectively, and not exceed the published limits for those cannabinoids in any demographic. In the present study, adipose 9-THC concentrations would need to be several fold higher to reach the most conservative published ARfD of 1 µg/kg 9-THC in most groups (EFSA, 2015). If using a less strict cutoff for ARfD, such as the 7 µg/kg threshold established in Switzerland and some other countries, 9-THC concentrations would need to be 4-fold higher than in the current study for the highest-exposure group (newborn males) (Beitzke and Pate, 2021). Adipose CBD concentrations would need to be approximately 26-fold higher than the present study to reach the 150 µg/kg/d ADI (Choudhry and Haynes, 2024). This hypothetical situation seems doubtful even at higher dietary IH inclusion levels. Irawan et al.

(2025) reported adipose CBD concentrations 28 d after final exposure to IH which would not result in intake above the ADI using our calculations, even if using the high end of the CBD estimate (1,456 ng/g). However, 9-THC concentrations in adipose at the end of the withdrawal period in that study (92 ± 107 ng/g) could still exceed the 1 µg/kg ARfD for some populations (up to 461% ARfD, using 199 ng/g 9-THC). Using a less conservative threshold for 9-THC ARfD, the concentrations reported by Irawan et al. (2025) at the end of the 28-day withdrawal would represent 66% of a 7 µg/kg ARfD for newborn males (the highest exposure group). Visually estimating 9-THC concentration at the end of the intervention period (no withdrawal) in Irawan's study to be 500 ng/g, this concentration would result in 165% and 150% of a 7 µg/kg ARfD for newborn males and females, respectively but would not result in exposures above 7 µg/kg in any other demographic.

Hempseed cake, SHB, or silage with low concentrations of cannabinoids appear to be safe feed sources for cattle (Wagner et al., 2022, Smith et al., 2023, Irawan et al., 2024b, Irawan et al., 2025). Considering the discussion above, it is plausible that a practical withdrawal period is attainable for cattle fed SHB or low amounts of unextracted plant material; however, tolerance limits are necessary for these future discussions. Comparison of plasma and tissue cannabinoid dispositions in cattle after exposure to various IH forms would be useful to explore potential bioavailability differences and would inform WDI recommendations. It is possible that dietary fiber, fatty acid, protein content, or dietary and rumen pH could impact cannabinoid absorption.

The tissue sampling schedule for this study, selected based on the published half-life of CBDA (14.1 h), presents some limitations (Kleinhenz et al., 2020a). While some depot formation was anticipated, the collection timepoints did not allow CBD concentrations in adipose to reach LOD. The WDI estimates are made assuming linear depletion of residues. However, it is likely

that there is a slower terminal elimination phase for CBD in adipose. The methods used in this study tend to overestimate WDI's to ensure consumer safety. However, the authors believe there is likely a separate terminal elimination phase for CBD in adipose that was not reached during the sampling schedule in this study. Even if the terminal elimination rate was the same as the depletion rate we were able to model with the data from this study, extrapolating from 8 days (final sample collection) to 154 days (WDI estimate) incorporates uncertainty into our models. The FDA recommends sampling to include at least one timepoint where tissue concentrations are detectable but below the tolerance (FDA CVM, 2022). Additional studies incorporating a longer sampling window are needed to precisely describe depletion times.

Some of our cannabinoid WDI estimates are lengthy, but establishment of tolerance limits for cannabinoids is required to interpret and apply WDIs. The ARfD used in our calculations has been criticized for being excessively strict and not evidence-based compared to other global thresholds for 9-THC exposure (Beitzke and Pate, 2021). An analysis by the German Federal Institute for Risk Assessment found that some hemp-derived foods may result in exposures beyond 1 µg/kg (BfR, 2018). However, in a review of the BfR analysis, authors from the nova-Institute concluded that the proposed ARfD was too stringent and that the safety factors applied to 9-THC were excessive and inconsistent compared with safety factors used for other food contaminants (Skoczinski et al., 2019). The authors cited multiple more recent studies regarding IH-derived foods that had not been considered by the BfR and which indicated the safety of IH products. (Do note that some of the authors of that review have ties to the European Industrial Hemp Association.) As discussed above, if using an ARfD greater than 1 µg/kg, it is possible that tissue concentrations from exposed cattle may be safe even with zero withdrawal.

There are multiple other countries that have adopted higher ARfD's for 9-THC that are worth consideration when establishing tolerance guidelines for cattle in the United States.

Conversely, there is concern that the 1 µg/kg ARfD may not be sufficient to protect vulnerable populations, particularly infants, toddlers, and adolescents. The EFSA assessment did not look at neurodevelopmental toxicity, so it is possible that the current ARfD would not account for those effects (EFSA, 2015). In fact, it was due to the lack of toxicity data in infants and children that the EFSA applied a higher safety factor to their calculations when deriving ARfD. Cannabinoid exposure in the perinatal (pre- and postnatal) and adolescent periods in rodent models and humans has been shown to result in behavioral and neurologic changes (Hurd et al., 2019). Exposure in this period of brain development alters neural plasticity and synaptic function which predispose these individuals to later psychiatric and abuse disorders and decreased cognitive ability. While young populations are particularly at risk, there are long-term cognitive and behavioral changes in adults and potential functional and structural changes with chronic exposure (NIDA, 2024). In addition, early cannabinoid exposure has been shown to disrupt metabolism and reproductive performance in rodents (Martínez-Peña et al., 2021). Variable doses in rodent models and exposures from human studies make it challenging to identify a precise dose at which these toxicities occur. Due to the unapproved status of IH or any cannabinoid product in cattle, any detectable concentration of cannabinoid in feed or tissue is considered an adulterant (FDA, 2011, 2019, AAFCO, 2022). We anticipate that our data will be useful in re-evaluating the risk associated with IH products in livestock feed. Based on the results presented in this study and data from previous studies in cattle and sheep, regulation of 9-THC and CBD content and total dietary percentage will be needed to ensure inclusion in livestock

feed is safe for human consumers (Smith et al., 2023, Irawan et al., 2024a, Fruge et al., 2025, Irawan et al., 2025).

This study spotlights several areas for future research. There are published data on the metabolic pathways for cannabinoids in humans, where CYP isoforms CYP2C19 and CYP3A4 are critical for CBD and 9-THC metabolism, with CYP2C9 additionally playing an important role for 9-THC (Jiang et al., 2011, Gaston and Friedman, 2017). One study investigating the effects of SHB on liver transcriptomics in cattle saw no changes in level of CYP isoform expression, but the authors did not compare activity of the different isoforms in the presence of individual cannabinoids (Irawan and Bionaz, 2024). Further studies elucidating the metabolic pathways of cannabinoids in cattle and the role of rumen microflora in cannabinoid conversion could help predict tissue disposition in exposed animals. In addition, comparing plasma and tissue residue profiles from cattle exposed to different IH products (e.g. inflorescence, hempseed cake, green hemp, SHB) would enable a nuanced regulatory approach to IH in livestock feed.

In summary, a variety of cannabinoids, including the psychoactive cannabinoid 9-THC and the bioactive cannabinoid CBD, were detected in tissues of cattle administered IH inflorescence. Tissue residue depletion models generated WDI estimates up to 154 d, but the sampling schedule was likely inadequate to accurately model the terminal elimination phase for cannabinoids in adipose. Human exposure calculations revealed one consumer group (newborns) exceeded the most conservative international toxicity threshold for 9-THC. Further work is needed to determine the toxicity level of cannabinoids in sensitive human populations (i.e. infants, children, and adolescents). Future regulatory discussions should focus on defining acceptable risk of exposure to 9-THC and CBD to enable consistent WDI recommendations for exposed cattle.

Funding and disclaimer

Data availability

Raw cannabinoid concentration data, sorted by tissue, can be viewed in Supplementary Tables S1-8 and FDA WithdrawalApp reports can be viewed in the Supplementary Reports R1-R24.

Figure 3.1. Withdrawal times of cannabinoids in liver, kidney, muscle, and adipose tissue of male Holstein cattle administered industrial hemp at a target dosage of 5 mg/kg cannabidiolic acid (CBDA) per day for 14 days.[4]

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[4] Total cannabidiol (CBD): CBD + (0.877*CBDA). Total Δ9-tetrahydrocannabinol (9-THC): 9-THC + (0.877*THCA); THCA: Δ9-tetrahydrocannabinolic acid A. Total cannabinoids: sum of all cannabinoids detected. CBD-7-acid: (−)-7-nor-7-carboxy cannabidiol. THC-acid: (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol. THC-glu: Δ9-tetrahydrocannabinol glucuronide.

Table 3.1. Cannabinoid content in industrial hemp (IH) inflorescence and average amounts (mg) and doses (mg/kg bodyweight) of cannabinoids administered to male Holstein cattle at a target dosage of 5 mg/kg cannabidiolic acid (CBDA) per day for 14 days.

Cannabinoid	Limit of detection by LCMS	Concentration in IH	Amount given	Dose
	mg/g	*mg/g*	*mg*	*mg/kg*
9-THC	0.2	1	11.36	0.04
THCA	0.2	2.3	26.14	0.09
THCV	0.2	N/D	-	-
CBC	0.2	1.3	14.77	0.05
CBD	0.2	11.1	126.14	0.43
CBDA	0.2	109.3	1,242.04	4.22
CBDV	0.2	N/D	-	-
CBG	0.2	11.6	131.82	0.45
CBGA	0.2	3.7	42.05	0.14
CBN	0.2	N/D	-	-

N/D – not detected
Additional details regarding dose calculation can be found in Supplementary Table S9.

Table 3.2. Cannabinoid concentrations (ng/g) in liver, kidney, muscle and adipose tissue of male Holstein cattle administered industrial hemp inflorescence at 4.22 mg/kg cannabidiolic acid (CBDA) per day for 14 days.

	Day after last dose[1]									
	1		2		3		5		8	
Analyte[2]	Mean[3]	SEM	Mean[3]	SEM	Mean[3]	SEM	Mean[3]	SEM	Mean[3]	SEM
Liver										
9-THC	3.25	0.79	2.66	1.01	N/D	N/D	0.41	0.41	N/D	N/D
THCA	61.18	12.82	25.98	5.42	7.58	2.67	5.24	2.54	1.5	N/A
THC-acid-glu	0.48	0.48	N/D	N/D	3.49	2.11	0.65	0.65	2.27	2.27
THC-glu	11.20	2.69	3.98	1.48	1.16	0.68	0.86	0.86	N/D	N/D
CBD	23.00	6.22	16.42	4.27	1.75	0.72	2.32	1.62	0.37	0.37
CBDA	37,339.86	24,739.28	4,376.99	1,970.97	966.07	465.63	1,131.30	1,086.84	11.28	7.17
CBDVA	12.25	1.30	2.96	1.07	N/D	N/D	0.62	0.62	N/D	N/D
CBLA	16.25	7.51	2.34	2.34	1.27	0.76	0.32	0.32	N/D	N/D
CBD-7-acid	135.62	31.61	225.53	57.46	93.14	16.37	128.16	60.68	49.38	8.39
Kidney										
8-THC	5.10	0.46	3.35	1.46	1.35	0.99	N/D	N/D	N/D	N/D
9-THC	7.65	1.02	7.02	2.60	4.67	1.88	0.66	0.39	1.69	0.57
THCA	7.80	1.18	4.39	0.79	1.11	0.38	N/D	N/D	N/D	N/D
THC-acid	20.83	7.20	28.55	5.19	20.73	4.81	5.18	5.18	N/D	N/D
CBD	9.27	1.86	11.55	4.70	7.96	3.46	1.84	0.66	3.15	1.10
CBDA	10.56	2.64	6.29	1.07	1.67	1.05	N/D	N/D	0.41	0.41
Muscle										
THC-acid	1.48	0.16	4.95	1.06	2.91	0.53	3.93	1.64	1.77	0.38
CBD	1.42	0.51	2.11	0.37	1.52	0.88	N/D	N/D	1.75	1.75
CBDA	18.79	6.36	7.96	2.31	2.97	2.17	N/D	N/D	0.34	0.34
CBD-7-acid	14.49	1.23	25.71	7.42	21.29	3.82	13.96	3.85	8.82	1.36
Adipose										
9-THC	46.76	3.58	59.08	6.13	43.79	4.31	43.74	3.99	28.06	5.87
CBD	223.37	25.66	287.09	32.45	203.66	35.29	243.83	38.25	142.86	44.33
CBDA	33.02	12.09	11.88	8.49	N/D	N/D	N/D	N/D	N/D	N/D
CBD-7-acid	79.38	27.35	115.43	49.59	105.06	13.34	53.73	15.57	54.65	18.44

[1]'Day after last dose' indicates the timepoints for humane euthanasia and tissue collection.
[2] 8-THC: Δ8-tetrahydrocannabinol; 9-THC: Δ9-tetrahydrocannabinol; THCA: Δ9-tetrahydrocannabinolic acid A; THC-acid: (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol; THC-acid-glu: (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide; THC-glu: Δ9-tetrahydrocannabinol glucuronide; CBD: cannabidiol; CBD-7-acid: (−)-7-nor-7-carboxy cannabidiol; CBLA: cannabicyclolic acid; CBDVA: cannabidivarinic acid.
[3]Mean of the four replicates (animals) from that day. Values < LLOQ but > LOD were included unchanged and samples with undetectable concentrations were included as '0'.
N/D – not detected in any replicates (animals) from that day; N/A – not applicable

Table 3.3. Withdrawal times of cannabinoids in liver, kidney, muscle, and adipose tissue of male Holstein cattle administered industrial hemp at a dosage of 4.22 mg/kg cannabidiolic acid (CBDA) per day for 14 days.

	Withdrawal time, days[1]			
	Tissue			
Cannabinoid[2]	*Adipose*	*Kidney*	*Liver*	*Muscle*
9-THC	49	-	-	-
THCA	-	2	6	-
Total THC	49	10	7	-
THC-acid	-	-	-	4
THC-glu	-	-	3	-
CBD	154	9	3	-
CBDA	-	-	15	4
Total CBD	106	15	15	12
CBD-7-acid	78	-	68	39
Total cannabinoids	104	13	19	22

[1]Withdrawal times were modeled in the FDA Withdrawal App in R Studio and were rounded up to the nearest whole day. To satisfy linearity and homoscedasticity requirements of the model, a withdrawal time was unable to be estimated for all cannabinoids detected in each tissue.
[2]9-THC: Δ9-tetrahydrocannabinol. THCA: Δ9-tetrahydrocannabinolic acid A. CBD: cannabidiol. Total cannabidiol (CBD): CBD + (0.877*CBDA); total Δ9-tetrahydrocannabinol (9-THC): 9-THC + (0.877*THCA); total cannabinoids: sum of all cannabinoids detected in that sample. CBD-7-acid: (−)-7-nor-7-carboxy cannabidiol. THC-acid: (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol. THC-glu: Δ9-tetrahydrocannabinol glucuronide.

Table 3.4. Limit of detection (LOD), lower limit of quantification (LLOQ), and upper limit of quantification (ULOQ) of cannabinoids in liver, kidney, muscle, and adipose tissues.

	Tissue											
	Liver			**Kidney**			**Muscle**			**Adipose**		
	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
Cannabinoid	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*	*ng/g*
8-THC	1.0	2.5	125	2.5	6.25	125	NE	NE	NE	2.5	10	1,000
9-THC	1.0	2.5	125	2.5	6.25	125	1.0	2.5	62.5	2.5	10	1,000
THCA	1.0	2.5	125	1.0	2.5	125	1.0	2.5	125	2.5	10	1,000
THCP	2.5	12.5	125	1.0	2.5	125	1.0	2.5	125	NE	NE	NE
THCV	1.0	2.5	125	2.5	6.25	125	1.0	2.5	125	2.5	10	1,000
THC-11-OH	1.0	2.5	125	NE	NE	NE	1.0	2.5	125	2.5	10	1,000
THC-acid	1.0	2.5	125	2.5	6.25	125	1.0	2.5	62.5	2.5	10	1,000
THC-acid-glu	1.0	2.5	125	1.0	2.5	125	1.0	2.5	125	NE	NE	NE
THC-glu	1.0	2.5	125	1.0	2.5	125	1.0	2.5	125	2.5	10	500
CBC	1.0	2.5	125	1.0	2.5	125	1.0	2.5	125	25	100	1,000
CBCA	1.0	2.5	62.5	1.0	2.5	62.5	1.0	2.5	62.5	2.5	10	1,000
CBD	1.0	2.5	125	2.5	6.25	125	1.0	2.5	125	2.5	10	1,000
CBDA	2.5	6.25	125	2.5	6.25	62.5	1.0	2.5	62.5	2.5	10	1,000
CBDV	1.0	2.5	125	2.5	6.25	125	1.0	2.5	62.5	2.5	10	1,000
CBDVA	2.5	12.5	125	1.0	2.5	62.5	1.0	2.5	62.5	NE	NE	NE
CBD-7-acid	1.0	2.5	125	2.5	6.25	125	2.5	6.25	125	2.5	10	1,000
CBG	1.0	2.5	125	2.5	6.25	125	1.0	2.5	62.5	2.5	10	1,000
CBGA	2.5	6.25	125	1.0	2.5	62.5	1.0	2.5	62.5	2.5	10	1,000
CBL	2.5	12.5	125	2.5	12.5	125	NE	NE	NE	2.5	10	1,000
CBLA	2.5	12.5	125	NE	NE	NE	1.0	2.5	125	2.5	10	1,000
CBN	1.0	2.5	125	2.5	6.25	125	1.0	2.5	125	2.5	10	1,000

NE – not established

Table 3.5. Exposure estimates in males for Δ9-tetrahydrocannabinol (9-THC) and cannabidiol (CBD) in beef fat from cattle administered industrial hemp inflorescence.

Age	BW[1]	Fat intake[2]	Cannabinoid intake[3]		Allowable exposure[4]		Intake/ allowable[5]	
			9-THC	CBD	9-THC	CBD	9-THC	CBD
y	*kg*	*g/d*	*ng/d*	*ng/d*	*μg*	*μg*	*%*	*%*
Newborn	3.41	79	5,901	29,309	3.41	512	173.1	5.7
0.5	8.41	79	5,901	29,309	8.41	1,262	70.2	2.3
1	10.59	125	9,338	46375	10.59	1,589	88.2	2.9
1.5	12.07	125	9,338	46375	12.07	1,811	77.4	2.6
2-3	13.14	121	9,039	44,891	14.0	2,100	64.6	2.1
3-6	16.6	136	10,159	50,456	16.6	2,490	61.2	2.0
6-11	23.9	168	12,550	62,328	23.9	3,585	52.5	1.7
11-16	46.5	223	16,658	82,733	46.5	6,975	35.8	1.2
16-21	71.3	278	20,767	103,138	71.3	10,695	29.1	1.0
21-31	85.5	254	18,974	94,234	85.5	12,825	22.2	0.7
31-41	94.4	352	26,294	130,592	94.4	14,160	27.9	0.9
41-51	93.9	218	16,285	80,878	93.9	14,085	17.3	0.6
51-61	91.9	214	15,986	79,394	91.9	13,785	17.4	0.6
61-71	91.2	197	14,716	73,087	91.2	13,680	16.1	0.5
71-81	87.7	144	10,757	53,424	87.7	13,155	12.3	0.4
81+	80.5	137	10,234	50,827	80.5	12,075	12.7	0.4

[1]Body weight (BW) values represent means for each sex and age group and were retrieved from the Centers for Disease Control (CDC), with most values extracted from anthropometric data from 2015-2018 and values for newborn through 2 y extracted from the most recent (2000) growth charts(Kuczmarski et al., 2002, Fryar et al., 2021). Where ages did not align with the fat intake groups, the BW associated with the youngest age for the intake range was used. For ages 21+, the BW of the most similar age group to the fat intake age group was used (e.g. for the 31-41 y intake group, BW from 30-39 y was used).
[2]Fat intake data (95th percentiles within males and females) of each age group were retrieved from Table 11-38 of the 2018 Environmental Protection Agency Exposure Factors Handbook representing the top decile of animal fat consumers(EPA, 2018). For age ranges 71-81 y and 81+ y, separate data were not available for males and females.
[3]Intake was calculated as (Fat intake in g/d) × (9-THC or CBD concentration in ng/g). Concentrations of 9-THC and CBD were the upper 99% CI from the day with the highest 9-THC or CBD concentrations in cattle adipose tissue (day 2): 74.7 ng/g and 371 ng/g, respectively.
[4]The allowable 9-THC exposure (1 μg/kg/d) is from the acute reference dose (ARfD) published by the European Food Safety Authority(EFSA, 2015). The allowable CBD exposure (0.15 mg/kg/d = 150 μg/kg/d) is from the acceptable daily intake (ADI) published by the Food Standards Agency (FSA)(Choudhry and Haynes, 2024).
[5]Intake/allowable was calculated as [(ng intake of 9-THC ÷ 1,000 ng/μg)] ÷ (μg allowable exposure of 9-THC) × 100% and [(ng intake of CBD) ÷ 1,000 ng/μg)] ÷ (μg allowable exposure of CBD) × 100%.

Table 3.6. Exposure estimates in females for Δ9-tetrahydrocannabinol (9-THC) and cannabidiol (CBD) in beef fat from cattle administered industrial hemp inflorescence.

Age	BW[1]	Fat intake[2]	Cannabinoid intake[3]		Allowable exposure[4]		Intake/ allowable[5]	
			9-THC	CBD	9-THC	CBD	9-THC	CBD
y	kg	g/d	ng/d	ng/d	μg	μg	%	%
Newborn	3.29	69	5,154	25,599	3.29	494	156.7	5.2
0.5	7.66	69	5,154	25,599	7.66	1,149	67.3	2.2
1	9.79	89	6,648	33,019	9.79	1,469	67.9	2.2
1.5	11.11	89	6,648	33,019	11.11	1,667	59.8	2.0
2-3	12.48	109	8,142	40,439	12.48	1,872	65.2	2.2
3-6	15.4	130	9,711	48,230	15.4	2,310	63.1	2.1
6-11	23.7	123	9,188	45,633	23.7	3,555	38.8	1.3
11-16	48.1	223	16,658	82,733	48.1	7,215	34.6	1.1
16-21	65.7	278	20,767	103,138	65.7	9,855	31.6	1.0
21-31	74.9	160	11,952	59,360	74.9	11,235	16.0	0.5
31-41	79.3	160	11,952	59,360	79.3	11,895	15.1	0.5
41-51	80.8	150	11,205	55,650	80.8	12,120	13.9	0.5
51-61	78.7	126	9,412	46,746	78.7	11,805	12.0	0.4
61-71	78.2	120	8,964	44,520	78.2	11,730	11.5	0.4
71-81	74.7	144	10,757	53,424	74.7	11,205	14.4	0.5
81+	67.9	137	10,234	50,827	67.9	10,185	15.1	0.5

[1]Body weight (BW) values represent means for each sex and age group and were retrieved from the Centers for Disease Control (CDC), with most values extracted from anthropometric data from 2015-2018 and values for newborn through 2 y extracted from the most recent (2000) growth charts(Kuczmarski et al., 2002, Fryar et al., 2021). Where ages did not align with the fat intake groups, the BW associated with the youngest age for the intake range was used. For ages 21+, the BW of the most similar age group to the fat intake age group was used (e.g. for the 31-41 y intake group, BW from 30-39 y was used).

[2]Fat intake data (95th percentiles within males and females) of each age group were retrieved from Table 11-38 of the 2018 Environmental Protection Agency Exposure Factors Handbook representing the top decile of animal fat consumers(EPA, 2018). For age ranges 71-81 y and 81+ y, separate data were not available for males and females.

[3]Intake was calculated as (Fat intake in g/d) × (9-THC or CBD concentration in ng/g). Concentrations of 9-THC and CBD were the upper 99% CI from the day with the highest 9-THC or CBD concentrations in cattle adipose tissue (day 2): 74.7 ng/g and 371 ng/g, respectively.

[4]The allowable 9-THC exposure (1 μg/kg/d) is from the acute reference dose (ARfD) published by the European Food Safety Authority(EFSA, 2015). The allowable CBD exposure (0.15 mg/kg/d = 150 μg/kg/d) is from the acceptable daily intake (ADI) published by the Food Standards Agency (FSA)(Choudhry and Haynes, 2024).

[5]Intake/allowable was calculated as [(ng intake of 9-THC ÷ 1,000 ng/μg)] ÷ (μg allowable exposure of 9-THC) × 100% and [(ng intake of CBD) ÷ 1,000 ng/μg)] ÷ (μg allowable exposure of CBD) × 100%.

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Chapter 4 - Pilot study: Impacts of cannabinoids from industrial hemp and repeated transportation events on cattle health and immune status[5]

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ABSTRACT

Recent legislative approval of industrial hemp (IH) cultivation has increased interest in the possibility of using IH and IH byproducts in livestock feed. Understanding the therapeutic effects of IH is critical for regulatory decisions and potential application to the cattle industry. The objective of this pilot study was to describe the effects of IH administration on stress and inflammatory biomarkers and activity in cattle experiencing repeated transportation. Twelve Holstein steers were randomly assigned to treatment sequences ($n = 3$ per sequence) in a 4x4 Latin Square design study (four periods and four treatment sequences). A 2x2 factorial treatment structure was used, with animals receiving one of two drugs (IH or placebo; HEMP, PLBO) and one of two transportation events (transport or control; TRANS, CNTL) during each round so that every animal received all four treatment combinations during the study. Industrial hemp was dosed at 5.5 mg/kg cannabidiolic acid (CBDA), with IH or placebo given once by oral bolus immediately prior to the transport or control event. Bodyweight, accelerometry, kinetic gait analysis, mechanical nociceptive threshold (MNT), infrared thermography (IRT), complete blood count (CBC), serum biochemistry, blood cortisol, prostaglandin E_2 metabolite (PGEM), serum amyloid A (SAA), and plasma cannabinoid concentrations were assessed. Data were analyzed using multiple linear regression. There was a drug by transport by time interaction for change in PGEM from baseline ($P = 0.03$). Cattle in the HEMP-CNTL group had negative changes in PGEM at 48 h compared to baseline, whereas PLBO-CNTL animals had positive changes at 48 h compared to baseline. During the transport period, TRANS cattle had increased activity as compared to CNTL ($P < 0.0001$). Both TRANS and CNTL steers had increased lying times in the period immediately after transport. Cattle in the TRANS group had significantly greater weight loss ($P < 0.0001$), neutrophils ($P < 0.0001$), monocytes ($P = 0.04$), blood glucose ($P <$

0.0001), and total protein ($P < 0.0001$) compared to CNTL steers immediately following transport. Cortisol area under the curve values were greater for TRANS versus CNTL steers ($P < 0.0001$). Lymphocyte concentrations were decreased for TRANS steers compared to CNTL following transport ($P < 0.0001$). There was a transport by time interaction for SAA ($P < 0.0001$), with TRANS cattle at 24 and 32 h having the highest SAA concentrations. Cannabidiolic acid reached the highest concentrations of all cannabinoids measured. Our results support previous transport literature regarding bodyweight, CBC, and inflammatory outcomes in cattle. The effects of IH on PGEM require further investigation. Novel outcomes evaluated in this pilot study will assist in design of future transportation trials.

Key words: cannabinoids, cattle, industrial hemp, inflammation, stress, transportation

Abbreviations

8-THC – Δ8-tetrahydrocannabinol

9-THC – Δ9-tetrahydrocannabinol

AUC – area under the curve

CBC – cannabichromene

CBCA – cannabichromenic acid

CBD – cannabidiol (CBD)

CBD-6-OH – 6-hydroxycannabidiol

CBD-7-acid – (−)-7-nor-7-carboxy cannabidiol

CBD-7-OH – 7-hydroxycannabidiol

CBDA – cannabidiolic acid

CBG – cannabigerol

CBGA – cannabigerolic acid

CBN – cannabinol

CBDV – cannabidivarin

CBDVA – cannabidivarinic acid

IH – industrial hemp

IRT – infrared thermography

LLOQ – lower limit of quantification

MNT – mechanical nociceptive threshold

PGEM - prostaglandin E2 metabolite

QC – quality control

SAA – serum amyloid A

THC-11-OH – (±)-11-hydroxy-Δ9-tetrahydrocannabinol

THC-acid – (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol

THC-acid-glu – (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide

THCA – Δ9-tetrahydrocannabinolic acid A

THCV – Δ9-tetrahydrocannabivarin

UPLC-MS/MS – ultra-performance liquid chromatography triple quadrupole mass spectrometry

INTRODUCTION

Despite transportation being recognized as one of the most stressful events for livestock species, such as cattle (Vogel et al., 2019), most cattle in North America are transported at least once, and sometimes more than 5 times within their lives (Schwartzkopf-Genswein and Grandin, 2019). A myriad of physiologic changes accompanies the stress of transport and have been reviewed elsewhere (Van Engen and Coetzee, 2018). Despite the welfare and economic implications of transport stress, Van Engen and Coetzee (2018) have reported that most

interventions are applied after the transport event and generally consist of broad treatment of a group of animals with antibiotics (metaphylaxis) or vaccination. There is a clear need to establish effective means of reducing transport-related stress and subsequent disease in cattle.

Distinction of industrial hemp (IH)—*Cannabis sativa* containing ≤ 0.3% Δ9-tetrahydrocannabinol (9-THC), the psychoactive cannabinoid in marijuana—from marijuana has enabled legal cultivation of IH in the United States (Johnson, 2021). Primary markets for IH and its fiber, seeds, oils, and cannabinoid extracts have garnered wide-reaching interest, from the construction sector to medicinal products (Kleinhenz et al., 2020b). Production of some IH byproducts, including hempseed products, oils, and cannabinoid extracts (e.g. cannabidiol, or CBD, oil), subsequently results in "waste" plant material. Cattle and other ruminants are efficient at digesting plant material and converting it into edible tissue products for consumers, such as milk and meat products. The favorable nutrient profile and economic and environmental sustainability of using IH as a livestock feed has sparked interest from the livestock industry.

While our lab has previous investigated the plasma cannabinoid profiles of cattle administered a single dose of IH (Kleinhenz et al., 2020a) and stress and inflammatory changes in cattle administered a short-term course of IH (Kleinhenz et al., 2022), there is no published work evaluating the therapeutic potentials of IH in cattle. The pharmacodynamic properties of some cannabinoids, including roles in anti-inflammatory, analgesic, and antioxidant pathways (Takeda et al., 2008, Pellati et al., 2018, Rodríguez-Muñoz et al., 2018), make IH an attractive target for transportation research.

The objective of this pilot study was to describe the effects of repeated transport events and IH administration on stress and inflammatory biomarkers and activity levels in cattle.

MATERIALS AND METHODS

Ethics statement and animal disposition

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4628). All study activities were conducted in conformity to requirements from the United States Department of Agriculture, the State of Kansas, and American Association for Accreditation of Laboratory Animal Care according to *The Guide for the Care and Use of Agricultural Animals in Research and Teaching (ADSA ASAS PSA, 2020)*. No enrolled animals were sold for human or animal consumption following trial termination.

Animals and housing

Twelve ($n = 12$) Holstein steers, 1.5 years of age and weighing ($\pm$ SD) 948 $\pm$ 86 lb (430 $\pm$ 39 kg), were enrolled in December 2021. Animals had been previously acclimated to the research facility and were group-housed in outdoor pens with access to shelter throughout the study period. Animals were fed a custom grain mix twice daily and had ad libitum access to grass hay and water via an automated watering device during the entire study. The pen area supplied per calf exceeded the guidelines established in the Guide for the Care and Use of Agricultural Animals in Research and Teaching.

Experimental design

Steers were blocked by weight, and animals within blocks were randomly assigned to a treatment sequence using a random number generator (Excel; Microsoft Corp., Redmond, WA) in a 4x4 Latin Square design study (four periods and four treatment sequences) with 3 steers per treatment sequence. A 2x2 factorial treatment structure was used, with treatment combinations including (1) transport and industrial hemp (IH) administration, (2) transport and placebo

administration, (3) control and IH administration, and (4) control and placebo administration [transport (TRANS), control (CNTL), IH administration (HEMP), placebo (PLBO)]. There were 4 rounds of the study, with treatment sequence set so that no animal underwent transportation during consecutive rounds of the experiment or more frequently than every 28 days. Animals underwent a 10-day washout period between rounds. Due to the obvious nature of the treatments (transport and drug administration), blinding of the investigators was not possible; all investigators analyzing data were also present at sample collection. Baseline samples were collected at -24 h, with 0 h being the time of transport and IH or PLBO administration. Additional samples were collected at 8, 24, 32, and 48 h. Steers in the TRANS groups were loaded onto a stock trailer immediately following IH or PLBO administration and were hauled for 623 mi (1017 km), or approximately 8 h. Steers in the CNTL groups were returned to their pen following IH or PLBO administration. A diagram of treatment sequences and study design is shown in Fig. 4.1.

Industrial hemp dosing

Prior to study initiation, the cannabidiolic acid (CBDA) content as a percentage of total IH weight was determined using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS/MS) and was used to calculate IH doses on an as-fed basis. Hemp was submitted for extended cannabinoid panel analysis by the Kansas State University Olathe Campus Postharvest Physiology Lab (DEA registration no: RK0682256). Hemp content is presented in Table 4.1. Cattle in the HEMP groups received IH at a dose of 5.5 mg/kg CBDA by oral bolus. Hemp flower material was finely chopped using a food chopper, weighed, and transferred into gelatin capsules. Animals were monitored after IH administration to ensure

capsules were consumed (not spit out). Animals in the PLBO groups received an oral bolus with chopped alfalfa pellets contained in a gelatin capsule.

Industrial hemp was grown and handled in keeping with licensing requirements under the Kansas Department of Agriculture Industrial Hemp Research Program (license numbers: KDA-0621466839 and KDA-0302873296). The cultivar Endurance HT was used (harvested October 2021).

Sample collection

Blood samples were collected 24 h prior to the start of each study round (baseline) and at 8, 24, 32, and 48 h. The 8 h timepoint was collected upon return of the TRANS groups. At each sampling timepoint, 17 mL of blood was collected via jugular or coccygeal venipuncture with an 18-gauge, 1.5-inch needle. Blood was collected into a 3 mL red-top tube (no additive), 2 mL EDTA tube, and two 6 mL heparinized tubes. Blood samples were stored on ice until transported back to the laboratory. The non-additive and EDTA tubes were submitted to the Kansas State Veterinary Diagnostic lab for serum biochemistry and complete blood count analysis according to American Association of Veterinary Laboratory Diagnosticians standards. The heparinized tubes were centrifuged at 1,500 G for 10 minutes and each plasma sample was aliquoted into 4 1.5 mL cryovials and stored at -80°C until analysis.

Weight

Steers were weighed using an in-chute scale (TruTest, Datamars, Mineral Wells, TX, USA) 24 h prior to study initiation and at 0, 8, 24, 32, and 48 h. Change in body weight from 0 h was calculated for analysis.

Accelerometry

IceQube accelerometer leg bands (IceRobotics, Edinburgh, Scotland, UK) were placed on the lateral aspect of the left hind limbs, just proximal to the metatarsophalangeal joint as recommended by the manufacturer directions. The accelerometers were placed one day before baseline (-24 h) data capture to allow steers to acclimate. Data capture was continuous throughout all treatment rounds. Data were downloaded at the 48 h timepoint of each round, with steps, standing bouts, lying bouts, and motion index recorded. To account for expected differences in the transport period and allow standardization across periods of different lengths, parameters were summed across the following time periods: -24 to 0 h, 0 to 9 h, 9 to 24 h, and 24 to 48 h. All parameters were then converted to an hourly basis by dividing the sum by the corresponding number of hours for that period.

Mechanical nociceptive threshold (MNT)

Mechanical nociceptive threshold (MNT; the minimum amount of force required to induce a withdrawal response) was collected on the lateral aspect of the coronary band on the lateral claw of both the left front and hind limbs, using methods described by Kleinhenz et al. (2017). Measurements were taken at 24 h prior to study initiation and at 8, 24, 32, and 48 h. A hand-held pressure algometer (Wagner Instruments, Greenwich, CT, USA) was used to apply force perpendicularly to the skin at a rate of approximately 1 kg/s. Force measurements were measured in triplicate for each limb at each timepoint and were recorded by a second investigator to eliminate measurement bias. If an output was the same for consecutive measurements, a new measurement was recorded until a unique force output was obtained. This was to reduce the chance of error with the algometer not recording a separate attempt. The mean of the three measurements was used for statistical analysis.

Kinetic gait analysis

A commercially-available pressure and force measurement system (Strideway, Tekscan, Inc, South Boston, MA, USA) was used to analyze the gait of steers at -24 h, 8, 24, 32, and 48 h. Video synchronization was used to ensure correct identification of footfalls in the research software (Strideway 7.70, Tekscan, Inc., South Boston, MA, USA). Using methods described by Coetzee et al. (2014), stance time, stride length, force, force-time integral (FTI), and pressure were assessed in all limbs; for the forelimbs, gait distance and gait velocity were also assessed.

Infrared thermography (IRT)

A thermography camera (TiX580 infrared camera, Fluke Corporation, Everett, WA, USA) was used to capture images of the medial canthus of the left eye and the left hind leg (at the level of the coronary band on the lateral claw) at -24, 8, 24, 32, and 48 h. The camera was calibrated prior to obtaining images and the ambient temperature and relative humidity were recorded from weather monitoring information for the area. The images were obtained by pointing the camera at the steer's head or coronary band at a 45° angle and distance of 0.5 m. Images were analyzed for maximum temperature using research grade software (Fluke Smartview 4.3, Fluke Corporation, Everett, WA, USA) by drawing a 2 cm circle over the target area. For the hind limb images, the target area was drawn to include equal parts haired skin and hoof wall. The steers were restrained in a chute with a head catch for image acquisition.

Blood cortisol

Serum cortisol concentrations were determined using a commercially available radioimmunoassay kit (MP Biomedicals, Santa Ana, CA, USA) following manufacturer specifications with minor modifications, as described previously (Martin et al., 2022b). The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL

manufacturer-supplied standards 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. Two quality controls (QCs) at 25 and 150 ng/mL were run at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a Wizard2 gamma counter (PerkinElmer, Inc. Waltham, MA, USA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, MyAssays Ltd., Brighton, East Sussex, UK) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a CV > 18% were re-analyzed, based on manufacturer recommendations. Inter- and intra-assay CV were 12.2% and 36.7%, respectively. The average LLOQ was 1.7 ng/mL. Average non-specific binding was 2.69%. Cortisol area under the curve (AUC) values for -24 to 24 h and -24 to 48 h were calculated using the trapezoidal method described by Lay et al. (1996); however, no baseline correction was included. Maximum concentrations (C_{max}) for each individual animal per round were identified. The maximum and minimum observed C_{max} were recorded. Using Excel, the C_{max} values were log-transformed, and the mean, standard deviation, and 95% CI were calculated for each treatment group and across treatment groups. These summary statistics were then back transformed for reporting purposes.

Blood prostaglandin E$_2$ metabolite (PGEM)

Prostaglandin E$_2$ metabolites (PGEM) were analyzed using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) following manufacturer specifications with minor modifications, as previously described (Martin et al., 2022b). Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were

incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. Supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (Labconco Corp., Kansas City, MO, USA) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, LLC, San Jose, CA, USA).

Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well (%B/B$_0$) of $\geq$ 80% or $\leq$ 20%. Any individual sample outside the standard curve, with a %B/B$_0$ outside the 20-80% range, or a CV > 15% were re-analyzed. If the CV exceeded 15% following re-analysis, the average of the CV values was recorded (samples were only re-analyzed twice). The inter-assay CV and intra-assay CV were 15.8% and 15.7%, respectively. The average LLOQ was 7.8 pg/mL. Data were analyzed using a commercially available data analysis tool (MyAssays Desktop).

Serum amyloid A

Serum Amyloid A (SAA) concentrations were determined in serum samples using a multispecies ELISA assay (Tridelta Development Ltd., Maynooth, County Kildare, IRE). Manufacturer specifications were followed, and samples were diluted as necessary. Absorbance was measured at 450 nm on a SpectraMax i3 plate reader. Raw data were analyzed using MyAssays Desktop software for concentration determination. Standard curves were plotted as a

4-parameter logistic curve. Samples with a CV > 15% were re-analyzed, based on manufacturer recommendations; if the CV exceeded 15% following re-analysis, the average of the CV values was recorded (samples were only re-analyzed twice). Inter-assay and intra-assay CV were 2.16% and 21.9%, respectively.

Plasma cannabinoid concentrations

Plasma cannabinoids were measured as previously described (Kleinhenz et al., 2020a). Briefly, all solvents used such as methanol, acetonitrile, isopropanol, and formic acid were LCMS grade. Individual cannabinoid standards were purchased as solutions in methanol (Cerilliant Corp., Round Rock, TX, USA), including: (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide (THC-acid-glu), (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-acid), (±)-11-hydroxy-Δ9-tetrahydrocannabinol (THC-11-OH), cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), cannabidiol (CBD), cannabidiolic acid (CBDA), Δ9-tetrahydrocannabinolic acid A (THCA), cannabigerolic acid (CBGA), cannabigerol (CBG), Δ9-tetrahydrocannabinol (9-THC), Δ8-tetrahydrocannabinol (8-THC), cannabichromene (CBC), Δ9-tetrahydrocannabivarin (THCV), cannabinol (CBN). Cannabinoid analogs used as internal standards included (±)-cis-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide-d_3 (THC-glu-d_3), cannabidiol-d_3 (CBD-d_3), Δ9-tetrahydrocannabinol-d_3 (9-THC-d_3), (±)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol-d_9 (THC-acid-d_9), (±)-11-hydroxy-Δ9-tetrahydrocannabinol-d_3 (THC-OH-d_3), and cannabichromene-d_9 (CBC-d_9). All cannabinoids standards were kept in the freezer at − 20°C.

On the day of analysis, plasma samples were thawed at room temperature. Plasma, internal standard mixture (200 ng/mL), and acetonitrile with 0.1% formic acid were combined to precipitate plasma proteins. Internal standard was not added to the negative controls. Following

vortexing and centrifugation, the supernatant was diluted with ultra-pure 18Ω water. Samples were then loaded onto a solid phase extraction plate using a nitrogen positive pressure manifold. Washes were performed with methanol:water (25:75) and eluted with acetonitrile:methanol (90:10). Eluates were diluted with water prior to analysis.

Cannabinoid analysis was performed using an Acquity H class UPLC and a TQ-S triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). Chromatographic separation was achieved using an Eclipse Plus C18 UPLC column (100×2.1 mm, 1.8 μ, Agilent Technologies, Santa Clara, CA, USA) heated at 55°C. The mobile phase consisted of a gradient of water containing 0.1% formic acid (A) and acetonitrile (B) as follows: 0 min: 60% B, 6.50 min: 86% B, 7.50–9 min: 100% B, 9.01-12 min: 60% B. The flow rate was set at 0.5 mL/min, injection volume was 5 μL, and the run time per sample was 12 min. Data acquisition was performed using electrospray ionization in positive and negative mode using multiple reaction monitoring. Linear regression with a weighting factor of 1/X was used and accepted if the coefficient of correlation R^2 was > 0.99. Calibration curves were linear from 0.1 to 100 ng/mL for all cannabinoids.

Statistics

Data analysis was performed in JMP Pro (Version 16.0, SAS Institute Inc., Cary, NC, USA) using the distribution function for cannabinoid summary statistics and multivariate multiple linear regression for all other outcomes. Drug, transport, time, and their two-way and three-way interactions were treated as fixed effects. Block, round, and animal within round were treated as random effects. For IRT parameters, ambient temperature was also included as a random effect. Significance was set *a priori* at $P \leq 0.05$. If the overall F-test for a response variable was significant, pairwise comparisons were performed using Tukey's Honest Significant

Difference adjustment for multiple comparisons, if there were more than 2 comparison groups.

Figures were made using GraphPad (GraphPad Prism, 10.0, La Jolla, CA, USA).

RESULTS

Bodyweight

Weight and weight change results are presented in Fig. 4.2 and Table 4.2. There was a transport by time interaction for weight and weight change ($P < 0.0001$), where TRANS steers at 8 h lost the most weight. Bodyweight for TRANS steers was different from baseline (both -24 and 0 h) subsequent timepoints through 48 h. On average, TRANS cattle lost about 5% bodyweight (calculated using least squares means for TRANS at 8 h and the 0 h mean bodyweight).

Accelerometry

A graphical representation of step count outcomes is presented in Figure 4.3. All accelerometric outcomes are summarized in Table 4.3. There were transport by time interactions ($P < 0.0001$) for motion index, step count, and standing ratio, where TRANS steers from 0 to 9 h having higher outcomes than other groups. Lying ratio had a transport by time interaction ($P < 0.0001$), where TRANS steers from 0 to 9 h had the lowest lying ratios. Both TRANS and CNTL animals had higher lying ratios and lower step counts, standing ratios, and motion indices from 9 to 24 h compared to other periods ($P \leq 0.05$). There was a transport by time interaction for lying bouts ($P < 0.0001$), where TRANS animals from 0 to 9 h and 9 to 24 had fewer lying bouts than CNTL animals from those intervals.

Mechanical nociceptive threshold (MNT)

Mechanical nociceptive threshold outcomes are presented in Table 4.2. There was a transport by time interaction for left front average MNT value ($P = 0.02$), where CNTL and

TRANS animals had lower baseline values than CNTL or TRANS animals at 32 or 48 h, CNTL animals at 8 h, or TRANS animals at 24 h. There was a time main effect for left hind average MNT value ($P < 0.0001$), where values progressively increased from -24 to 48 h, with -24 and 8 h values being lower than all other timepoints ($P \leq 0.05$). The same temporal pattern was seen for the change in MNT values from baseline for both hind and front limbs ($P < 0.0001$), with greater positive percent change from baseline with increasing time.

Kinetic gait analysis

Kinetic gait analysis outcomes are presented in Table 4.4. There was a drug by transport interaction ($P = 0.04$) for rear pressure. However, post-hoc comparisons did not reveal any significant differences between groups. All outcome parameters except front and rear stance and front pressure had a time main effect ($P \leq 0.009$), with 8 h outcomes being the highest and 48 h being the lowest, except for front gait distance, in which 32 h outcomes were the highest.

Infrared thermography

Infrared thermography outcomes are summarized in Table 4.2. The percent change from baseline in left eye maximum temperature had a drug by transport interaction ($P = 0.008$), where HEMP-TRANS steers had greater positive change than HEMP-CNTL and PLBO-CNTL groups ($P \leq 0.05$). All IRT parameters had time main effects ($P \leq 0.009$), but there was no pattern shared among the parameters regarding the hierarchy of least squares means.

Complete blood count (CBC) and serum biochemistry

Select parameters, determined to be relevant to stress and transport, are shown in Table 4.5. Figure 4.4 shows white blood cell concentrations, segmented neutrophil concentrations, blood glucose, and total protein values over time for TRANS versus CNTL animals. Blood glucose had a drug by transport by time interaction ($P = 0.03$), where PLBO-TRANS and

HEMP-TRANS steers at 8 h had higher values than other groups and timepoints ($P \leq 0.05$). A contrast between the PLBO-TRANS and HEMP-TRANS groups was not significant ($P = 0.054$). White blood cell, neutrophil, monocyte, and lymphocyte concentrations, hematocrit, and total protein all had transport by time interactions ($P \leq 0.04$). White blood cell, neutrophil, and monocyte concentrations, and total protein were greater for TRANS cattle at 8 h; lymphocyte concentrations were the lowest for TRANS cattle at 8 h. For hematocrit, CNTL animals at 8 h had lower values than TRANS cattle at 8 and 48 h and CNTL cattle at -24 and 48 h ($P \leq 0.05$).

Cortisol

Results for average cortisol concentration and cortisol area under the curve (AUC) for -24 to 24 h and -24 to 48 h are shown in Table 4.6. Cortisol AUC data for TRANS versus CNTL animals are also presented in Fig. 4.5. There were transport and time main effects for average concentration ($P = 0.002$ and < 0.0001, respectively), where TRANS cattle had higher concentrations than CNTL. Average cortisol was higher for 8 and 24 h compared to all other timepoints, with -24 and 48 h having the lowest concentrations. There were transport main effects for the AUC parameters ($P < 0.0001$), where TRANS cattle had higher AUC values than CNTL. The minimum and maximum observed cortisol C_{max} were 0.39 and 17.79 ng/mL, respectively. The mean C_{max} across groups was 3.87 ng/mL (95% CI: 2.92, 5.14 ng/mL). The mean C_{max} values (95% CI) for HEMP, PLBO, TRANS, and CNTL animals were 3.46 ng/mL (2.26, 5.30 ng/mL), 4.33 ng/mL (2.98, 6.31 ng/mL), 6.58 ng/mL (5.14, 8.44 ng/mL), and 2.28 ng/mL (1.51, 3.44 ng/mL), respectively.

Prostaglandin E₂ metabolite (PGEM)

The results of PGEM analysis are shown in Table 4.6. There was a time main effect for PGEM concentration ($P = 0.02$), where values at 48 and -24 h were higher than at 8 h. There was

a drug by transport by time interaction for percent change from baseline ($P = 0.03$). Sliced ANOVA revealed this interaction was due to the contrast between PLBO-CNTL animals and HEMP-CNTL animals at 48 h, with the PLBO-CNTL animals having a higher positive change (31% versus HEMP-CNTL animals -9.3%).

Serum amyloid A

Serum amyloid A concentrations are represented in Table 4.6; Fig. 4.5 includes a graph of SAA concentrations over time for CNTL and TRANS groups. There was a transport by time interaction ($P < 0.0001$), where TRANS cattle at 24 and 32 h had the highest concentrations and had higher SAA than TRANS cattle at baseline ($P \leq 0.05$).

Cannabinoid concentrations

The lower limit of quantification and inter-day accuracies for each cannabinoid analyte are summarized in Table 4.7. Average (median) cannabinoid concentrations at each timepoint are summarized in Table 4.8. Four cannabinoids – CBD-7-acid, CBDA, CBDVA, and THCA – were consistently detected across timepoints and rounds. A total of 14 cannabinoids were detected above LLOQ. Cannabidiolic acid reached the highest concentrations, followed by CBD-7-acid, THCA, and CBDVA. The modal time for observed peak concentration was 48 h for CBD-7-acid, 24 h for CBDA and CBDVA, and 32 h for THCA. One steer was responsible for all positive CBD-6-OH, CBD-7-OH, THC-11-OH, and THC-acid samples. Similarly, another steer was solely responsible for all positive CBG, CBN, 9-THC, 8-THC, and CBC samples; this steer and one other were the only animals with positive CBGA samples. The cannabinoids CBC, CBD-7-acid, CBDA, CBDVA, CBG, CBN, 8-THC, 9-THC, and THCA were detected in some baseline (-24 h) samples. All six HEMP animals from round 3 had detectable CBD-7-acid at baseline, and

four out of six animals from round 4 had detectable CBD-7-acid at baseline, compared to only one positive animal in each of rounds 1 and 2.

DISCUSSION

This study is the first to report on the effects of concurrent IH administration and transport in cattle. We evaluated a wide variety of outcome measures to generate data for development of further studies investigating IH administration during transport. We found significant drug by transport interactions for the kinetic gait parameter, rear pressure, and IRT. We found significant drug by transport by time interactions for blood glucose and PGEM. There were transport by time interactions for a variety of outcome variables, including weight change, accelerometry, MNT, complete blood count and serum biochemistry, cortisol, and SAA concentrations.

We reported a significant transport by time interaction for weight change, with TRANS cattle not yet returning to their baseline (-24 or 0 h) weight by the final collection point at 48 h. Our results are consistent with bodyweight loss due to dehydration reported in previous transportation literature: cattle tend to lose 1% bodyweight per hour for the first few hours of transport and less thereafter (Coffey et al., 2001), lose the most weight within the first 12 hours of initiation of transport (Knowles, 1999, Knowles et al., 1999), and generally return to baseline bodyweight within 24 to 72 h following transport (Knowles et al., 1997). While the TRANS cattle in the present study had not returned to baseline weight by 48 h, there was an upward trend in weight change (less negative change, or increased weight) from 8 to 48 h.

Our accelerometric parameters support prior research that has observed increased lying times in transported heifers compared to controls (Theurer et al., 2013). Conversely, studies in bulls have reported decreased lying times (Earley et al., 2013), potentially in favor of interacting

with other animals (Cafazzo et al., 2012). Steers were used in the present study. While our data suggest that steers appear to behave more similarly to heifers than bulls, other characteristics of our study population (e.g. familiarity with people, breed) could also be responsible for the behaviors observed. It is interesting to note that, while TRANS animals laid down more compared to baseline—similar to the findings from Theurer et al. (2013)—in the present study, we also observed increasing lying times in the CNTL animals. While CNTL animals did follow a similar pattern to TRANS animals regarding motion index, step count, standing ratio, and lying ratio, CNTL animals had more lying bouts than TRANS cattle. Thus, TRANS animals appear to stay in recumbency longer, whereas CNTL animals appear to shift between standing and recumbency. Previous studies have not reported this apparent mimetic behavior in non-transported cohorts. All animals in the present study had been housed together for a prolonged period prior to this experiment; it is possible that the social dynamics between the animals in the present study account for the changes we observed in the CNTL cattle following return of the TRANS cohort. The increased lying bouts in CNTL compared to TRANS animals suggests that CNTL animals may have been more comfortable or less fatigued, and thus more willing to expend energy standing.

To the authors' knowledge, there are no reports of MNT outcomes in transported cattle. Our results suggest that cattle acclimate to MNT measurement over time. The observed temporal pattern of decreasing sensitivity over time is consistent with habituation to the stimulus (Grandin, 1997). While sensitivity generally decreased with time (MNT values increased), left front limb MNT values for TRANS cattle at 8 h were not different from baseline. One explanation for this delayed habituation in TRANS cattle is the possibility of tissue damage secondary to transport, which has been previously reported (Jarvis et al., 1996, Huertas et al., 2010). Tissue damage and

subsequent release of prostaglandins and other inflammatory mediators may initiate a state of hyperalgesia or sensitize nerve endings in the skin or deeper tissues (Grandin et al., 2023). Pressure algometry has previously been used to quantify pain due to lameness in livestock (Mohling et al., 2014, Kleinhenz et al., 2019, Weeder et al., 2023), in addition to evaluating other inflammatory or painful conditions—such as mastitis, dehorning, and branding (Heinrich et al., 2010, Fitzpatrick et al., 2013, Stock et al., 2016, Martin et al., 2022a). However, it has not gained attention in the transport literature. It is worth noting that not all MNT parameters had significant transport effects. Because this study evaluated a large number of outcomes, it is possible that the significant transport effects we observed for left front average MNT values were actually Type I errors. In addition, further work would be needed to distinguish the temporal pattern in MNT values from the effects of transport, due to the fact that the transport period was early in the study period, when MNT outcomes were already lower than later timepoints.

This is also the first report of kinetic gait analysis being used for evaluation of transport effects. Kinetic gait analysis has previously been used to evaluate the efficacy of analgesics in cattle with induced lameness; Schulz et al. (2011) observed that cattle treated with meloxicam placed greater force on both lame and non-lame limbs compared to placebo animals. In the present study, we observed time effects for most parameters. One explanation for the time effects is that cattle were becoming habituated or more resistant to handling at 48 h than earlier in the study, resulting in shorter, slower strides with less force. Introducing cattle to the pressure mat system prior to baseline collection would allow a more direct comparison of values throughout the study. Further work is needed to determine the utility of kinetic gait analysis subsequent to transportation.

Infrared thermography has been used previously to evaluate the effects of transportation. Lei et al. (2023) investigated non-invasive markers of stress in Arouquesa cattle and found increased mean and maximum eye IRT values immediately after transport, but temperatures subsequently returned to baseline following a short rest. One other potential use of IRT in transported animals is for early identification of those with respiratory disease (Schaefer et al., 2007, Schaefer et al., 2012, Marquez et al., 2024). Sustained elevations in IRT would likely not be indicative of transport stress but may indicate an underlying disease process. Brief elevations in IRT values could also be caused by elevated ambient temperatures within the transport vehicle. In the present study, left eye maximum temperatures were highest at 8 h, but this is more likely explained by time of day than transport stress, since there was no transport by time interaction. We observed a drug by transport interaction in the present study, in which HEMP-TRANS cattle had higher eye temperatures than HEMP-CNTL or PLBO-CNTL animals; however, this interaction was not significant for any other outcomes. Due to the number of outcomes analyzed, this may represent a Type I error, rather than a true effect of drug or transport condition.

We observed several changes to complete blood count and serum biochemistry parameters that are consistent with previous transportation literature, including an elevation in total white blood cell, neutrophil, and monocyte concentrations and a decrease in lymphocyte concentrations. Numerous publications have reported transport-induced neutrophilia, which is due to elevation in glucocorticoids causing decreased margination and expression of extravasation proteins (Murata et al., 1987, Earley et al., 2006, Earley and O'Riordan, 2006, Buckham Sporer et al., 2008, Hulbert et al., 2011, Van Engen and Coetzee, 2018). However, neutrophil concentration does not necessarily correlate with decreased function. Some authors

have reported decreases in phagocytic ability (Burdick et al., 2011), while others report increases in phagocytosis and oxidative burst capacity (Hulbert et al., 2011). There may also be a temporary inhibition and subsequent rebound of neutrophil oxidative enzyme activity (Murata et al., 1987). Similar to neutrophils, monocyte concentrations were also increased in TRANS animals at 8 h in the present study. The existing reports on monocyte concentrations following transport vary. Masmeijer et al. (2019) reported monocytosis in lightweight, but not "normal" weight, calves following transport. In a study evaluating the effects of meloxicam on stress biomarkers in transported bulls, both control and meloxicam groups exhibited elevated monocyte concentrations following transport compared to baseline (Van Engen et al., 2014). Other reports observed no change in monocyte levels (Gupta et al., 2007, Ishizaki and Kariya, 2010). It is possible that age, breed, or handling differences could be responsible for the disparity in reports on monocyte concentrations. Contrary to neutrophils and monocytes, lymphocyte concentrations were reduced in TRANS animals in the present study. Previous work has also demonstrated this reduction, which is glucocorticoid-mediated (Murata et al., 1987, Earley et al., 2006, Earley and O'Riordan, 2006). Some previously published transportation literature has focused on the impact of transportation on immune function. As the present study was a pilot study, we chose to only focus on immune cell numbers. It should be noted that white blood cell, neutrophil, and monocyte concentrations in all calves in the present study remained within the reference interval for our laboratory. Evaluation of the effects of transport on immunological function when IH is administered would be an area of future interest, considering the potential anti-inflammatory effects of some cannabinoids (Pellati et al., 2018, Cosentino et al., 2023).

Not only do glucocorticoids alter immune cell concentrations, but they have also been shown to transiently increase blood glucose concentrations in transported cattle (Kenny and

Tarrant, 1987, Warriss et al., 1995, Browning and Leite-Browning, 2013). In the present study, we observed mild hyperglycemia in all cattle, with PLBO-TRANS cattle at 8 h having the highest levels. While the trends we observed do match previous transport literature, the small numerical differences observed here are likely biologically insignificant and reflect the fact that these steers were well-accustomed to people and handling. Kenny and Tarrant (1987) noted variable glucose responses to loading and unloading, confinement in a stationary trailer, and transport in a moving trailer. In that study, glucose levels were highest for transported cattle and lowest for those loaded and unloaded. The difference in glucose responses was presumably due to the hierarchy of stress in the three scenarios, with loading and unloading being the least stressful. Similarly, the previous acclimation to handling may have dampened the immune cell and glucose responses of the steers in the present study, as habituation may have made study procedures less stressful. Although we did find a drug by transport by time interaction, the lack of biological significance suggests blood glucose is likely of low interest for future IH research.

While the elevations in immune cells and glucose point to transport-mediated stress, the increases we observed in total protein support existing data on dehydration during transport (Jarvis et al., 1996). The transport distance used in the present study was chosen to be an intermediate transport period. In the study by Jarvis et al. (1996), many cattle were transported for much shorter distances. The authors found that cattle transported less than 80 mi had lower packed cell volume and total protein than those transported more than 80 miles. It has also been observed that cattle lose more weight in high ambient transport temperatures than cooler conditions due to thermal stress (Coffey et al., 2001, Theurer et al., 2013). Total protein did not fall outside the reference interval for any animal in the present study. It is possible that, despite

the prolonged transport distance and time without water, the changes in total protein were mitigated by the cold temperatures.

Thus far, we have reviewed parameters affected by glucocorticoids (e.g. white blood cells and glucose). Those data are supported by our results for cortisol analysis, in which we found significant transport and time effects. We evaluated average cortisol concentrations and cortisol AUC, a measure of total cortisol exposure. Average concentrations were significantly higher in TRANS cattle than CNTL and at 8 and 24 h compared to baseline. Cattle in the TRANS groups also had significantly higher AUC than CNTL animals. This stress response to transport is well-established in the literature. Buckham Sporer et al. (2008) reported a 321% increase in cortisol concentration 4.5 h into a 9 h transport period; cortisol returned to baseline by 24 and 48 h following transport. In a multiple experiment trial evaluating the effects of transport, novel environments, and loading, authors reported cortisol increased in response to transport, introduction to a novel environment, and loading (Browning and Leite-Browning, 2013). That study demonstrated the multifactorial nature of transport stress, in that many aspects of typical cattle processing (loading, transport, new environment), all induced a cortisol response. The authors did not find a difference in cortisol response between cattle that were loaded and unloaded versus transported, suggesting those events are similarly stressful. In the present study, steers were returned to their home environment, so they were subjected only to the stress of loading and transport. The logarithmic mean concentrations observed in our study for -24, 32, and 48 h were less than 1 ng/mL, and the highest mean concentration (averaged across treatments) was observed at 8 h (3.06 ng/mL, 95% CI: 1.83 to 5.12). Past transport studies have generally reported baseline values between roughly 2 and 15 ng/mL, with substantial increases following transport (Kenny and Tarrant, 1987, Gupta et al., 2007, Buckham Sporer et al., 2008,

Browning and Leite-Browning, 2013). In our previous study evaluating the effects of short-term IH feeding (Kleinhenz et al., 2022), hemp-fed Holstein steers had a mean cortisol concentration of 1.59 ng/mL, whereas controls had a mean concentration of 5.97 ng/mL; the hemp group had cortisol concentrations as low as 0.76 ng/mL on the last day of feeding. While we were still able to identify significant effects of transport and time, the consistently low cortisol concentrations may reflect the habituation of the cattle in the present study to people and manipulation. (The authors also believe that the low cortisol concentrations observed in this study are responsible for the large intra-assay CV, as CV increases with decreasing mean values.) Van Engen et al. (2014) reported a reduction in cortisol following long-distance (16 h) transport and postulated that the decrease was due to acclimation to the stressor. Lay et al. (1996) evaluated the effects of transport, ACTH injection, or a sham on cortisol levels in pregnant Brahman heifers; the study was repeated at five points during gestation. Authors reported lower cortisol concentrations with the later transportation events and suggested that these lower values were due to acclimation to transportation. While the cattle in the present study weren't acclimated to transport, the frequent prior exposure to handling and people could have impacts on the magnitude of cortisol response to any novel stressor. Burdick et al. (2010) reported on the effects of temperament of Brahman bulls on physiologic responses to transport and found that bulls scored as 'calm' had lower pre- and post-transport cortisol concentrations than bulls scored as 'temperamental'. Considering that cortisol values from the previous study by Kleinhenz et al. (2022) were also low (albeit those cattle did not undergo transport), it is possible that there is some breed or bloodline temperament effect on cortisol levels consistent across the animal source used for our studies.

While there are minimal reports of cortisol AUC in the transport literature, our TRANS and CNTL cortisol AUC values are higher than those reported by Coetzee et al. (2008) when

evaluating the effects of castration or simulated castration on cortisol and substance P responses. In that study, cortisol AUC values from castrated animals were not statistically different than controls (castrated cortisol AUC: 137.87 h*nmol/L ± 6.11, which is equivalent to 49.97 h*ng/mL ± 2.21; molecular weight of cortisol: 362.46 ng/nmol). However, the animals in that study were 4 to 6 mo Angus-cross calves, limiting our ability to directly compare the cortisol response of transport versus castration between that study and the present study. While our AUC values were higher than reported in castrated calves, our C_{max} values are similar or lower than prior transportation literature. Kenny and Tarrant (1987) reported the highest mean cortisol levels in transported steers (32.1 ng/mL) compared to cattle that were loaded and unloaded (10.4 ng/mL) or confined to a truck (10.0 ng/mL) for equivalent lengths of time. In a study evaluating the effects of transport and space allowance in bulls, Gupta et al. (2007) reported the highest median cortisol levels in transported bulls (11.31 ng/mL), with maximum observed concentrations of 82.62 ng/mL. Buckham Sporer et al. (2008) observed maximum cortisol levels of 42.54 ng/mL after 4.5 h of a 9 h transport event in bulls (baseline cortisol: 13.22 ng/mL). In a multiple phase trial, Browning and Leite-Browning (2013) compared the effects of transport, fasting, and loading in Brahman and Hereford steers. They found that transport resulted in higher maximum cortisol concentrations when compared to fasting alone (17.58 vs. 9.03 ng/mL, respectively) and compared with unloading and loading or fasting groups (41.62 ng/mL transported; 38.01 ng/mL loaded/unloaded; 8.33 ng/mL fasted). The authors observed the highest cortisol levels immediately after transport (40.6 ng/mL) compared to the day before or after (< 12.6 ng/mL) (Browning and Leite-Browning, 2013). Summarizing this prior literature, maximum cortisol values following transport range from 11.31 to 82.62 ng/mL, with most studies citing maximum concentrations around 30 to 40 ng/mL. The highest mean C_{max} values from the present study was

6.58 ng/mL in the TRANS cattle, with a maximum observed cortisol value of 17.79 ng/mL. As discussed earlier, extensive prior handling likely impacted cortisol levels in the animals we used, with breed and bloodline potentially contributing as well. Despite the low values we report in this study, we were still able to confirm that transport induces a cortisol response. While we did not find a statistical difference for drug (IH) in this study, HEMP cattle had numerically lower cortisol AUC values than PLBO animals. Since our previous work showed that hemp-fed steers had lower cortisol than controls (Kleinhenz et al., 2022), the authors believe it would be worthwhile to continue investigation of the effects of IH on cortisol levels with a larger cohort or in other stressful management scenarios.

To the authors' knowledge, this is the first report of PGEM data from transported cattle. We observed an overall negative change in PGEM concentration in HEMP-CNTL cattle compared to a positive change in PLBO-CNTL cattle at 48 h. In a previous study, in which cattle were fed IH or a control for 14 d, we reported an 8.8% reduction in PGEM concentrations compared to baseline for cattle receiving hemp versus a 10.2% increase for control cattle (Kleinhenz et al., 2022). Previous work has shown that CBD and an IH water extract have inhibitory effects on PGE_2 production in animal and *in vitro* models of inflammation (Costa et al., 2004, Di Giacomo et al., 2021). Furthermore, cannabinoids, including CBD, CBDA, THCA, CBG, CBGA, and IH leaf and inflorescence extracts have been shown to decrease cyclooxygenase production in *ex vivo* and *in vitro* inflammatory models (Costa et al., 2004, Ruhaak et al., 2011, Shin et al., 2024). Specifically, THCA, CBG, CBGA have been shown to have COX-2 inhibitory properties (Ruhaak et al., 2011); results for CBDA have been mixed, with some studies showing inhibition (Takeda et al., 2008) and others showing an increase in COX-2 (Ruhaak et al., 2011). Bartkowiak-Wieczorek et al. (2025) showed dose-dependent

increases in COX-1 expression in rat brain following administration of two IH extracts and variable changes in COX-2 expression based on brain region. The effect observed in this study, complicated by the three-way interaction, warrants further work to confirm the effects of PGEM in cattle.

We evaluated two acute phase proteins (APP) in the present study. Past research has focused on the APP response to transport stress and as a potential early indicator of bovine respiratory disease (BRD). As an indicator of general inflammation, APP have poor specificity and low diagnostic value for early detection of BRD when used alone; however, they may have utility when used with other outcomes (Van Engen and Coetzee, 2018). In a study evaluating the effects of transport and commingling on APP in newly weaned calves, Arthington et al. (2003) found a tendency for SAA to be increased in transported calves (48.9 vs. 33.4 µg/mL in nontransported calves). However, these results are not straightforward to interpret, as there was not an unweaned control group. Thus, the relative contribution of transport versus weaning on the APP response in these calves is unknown. In a study by Lomborg et al. (2008), Holstein cows and heifers were subjected to a combination of transport, social isolation, and an uncomfortable environment. The authors reported significantly higher SAA in all animals at 48 h compared to 0 h, with a range of 30 to 482 mg/L SAA at 48 h. While we did see a transport by time interaction in the present study, all SAA values were much lower, with the highest average concentration in TRANS cattle at 24 h (20.8 µg/mL, 95% CI: 8.3 to 52.3). As discussed previously, these lower values could be due to the level of prior human socialization of the cattle in the present study. Another possible explanation is that there are differences in baseline APP profiles or the APP response to stress due to age, sex, or breed. We did not observe an effect of drug (IH) on SAA concentrations in the present study, consistent with our previous investigation of stress

biomarkers after a 14 d period of feeding IH (Kleinhenz et al., 2022). However, in both the present study and the study by Kleinhenz et al. (2022), animals were free of disease or other diagnosed inflammatory conditions. It is unknown if IH may impact SAA or other APP responses in light of a true inflammatory impetus.

Unlike SAA, we found no significant effects for fibrinogen. Previous literature has revealed mixed results for fibrinogen following transport. Arthington et al. (2003) found a significant day by transport interaction for fibrinogen, where values were higher in the transported group on days 1 and 3. Earley et al. (2012) found significantly higher fibrinogen levels in transported heifers compared to controls at unloading after an initial period of road and sea transportation. However, other studies have found no change (Van Engen et al., 2014) or a decrease (Buckham Sporer et al., 2008) in fibrinogen following transport. Considering the inconsistency of prior reports on post-transport fibrinogen and the insignificant changes we observed in the present study, the authors feel that it is not a useful measure of transport stress.

In the present study, we consistently detected 4 cannabinoids, CBD-7-acid, CBDA, CBDVA, and THCA, above the LLOQ. In a previous study investigating the pharmacokinetics of cannabinoids in cattle following a single oral dose of IH, our group detected CBDA, THCA, CBDVA, and CBCA in all samples and CBD in a few samples (Kleinhenz et al., 2020a). In a second study investigating a 14-d IH feeding period on plasma cannabinoid concentrations and effects on behavior and stress in cattle, we detected CBDA, CBD, CBDVA, CBCA, CBGA, and THCA (Kleinhenz et al., 2022). Smith et al. (2023) detected sporadic and low concentrations of CBDA/THCA, CBDVA, and CBNA in plasma samples from heifers fed hempseed cake for 111 days. The quantification methods used in that study were unable to differentiate between CBDA and THCA. In a recent study evaluating the depletion of cannabinoids in cattle fed IH leaves,

Fruge et al. (2025) detected CBD-7-acid, CBD, CBDA, CBDVA, THCA, and THCVA. Irawan et al. (2025) detected CBC, CBCA, CBD, CBDA, CBG, 9-THC, THCA, and THC-11-OH in plasma of cows fed spent hemp biomass. Based on visual inspection of the data from the studies by Kleinhenz et al. (2020a, 2022), Fruge et al. (2025), and Irawan et al. (2025), the maximum observed THCA concentrations in the present study are comparable. The CBDVA concentrations in the present study are similar to those reported by Kleinhenz et al. (2020a, 2022), but lower than those reported by Fruge et al. (2025). In the present study, we observed lower CBDA concentrations than the studies by Irawan and Fruge, but higher CBDA concentrations than reported by the previous Kleinhenz studies [72.7 ng/mL (Kleinhenz et al., 2020a) and 22 ng/mL (Kleinhenz et al., 2022) vs. 136 ng/mL – highest observed median concentration – in the present study]. The IH doses used by Kleinhenz et al. (2020a, 2022) and Fruge et al. (2025) were ~5.5 mg/kg CBDA and 3.9 mg/kg CBDA, respectively. The modal time of our peak CBDA concentrations (24 h) is later than the time to maximum CBDA concentration previously reported [11.8 h (Kleinhenz et al., 2020a) and 16.7 h (Kleinhenz et al., 2022)]. Fruge et al. (2025) reported maximum observed concentrations of CBD-7-acid at 72 h, as compared to 48 h in the present study. That study involved 14 days of IH leaf administration, but plasma was only collected for the first 72 h. Because the present study was not designed to determine pharmacokinetic parameters and we only took samples through 48 h, the timepoints that we observed maximum cannabinoid concentrations may not actually be the time of the true peak concentrations.

The varying cannabinoid signatures and pharmacokinetic profiles in the present study compared to previous work could be due to cultivar differences across studies. However, it is also possible that altered rumen pH or other physiological parameters could play a role in

changing cannabinoid absorption. Fruge et al. (2025) reported large variability in fecal cannabinoid profiles in cattle fed IH leaves, postulating that there were marked differences in individual animals' capacity to absorb or metabolize cannabinoids. The different cannabinoid signatures across studies could be due to individual animal differences. In addition, individual variability in metabolic or absorptive capacity could explain why some cannabinoids (including 9-THC) were only detected in one or two animals in the present study. Further investigation is needed to establish pharmacokinetic data for individual cannabinoids and IH forms and to illuminate the cause of the reported variation in individual animals' cannabinoid profiles.

We reported CBD-7-acid concentrations in the present study. This cannabinoid has only been reported in one other study, which evaluated cannabinoid depletion in cattle fed IH leaves (Fruge et al., 2025). Although considered an inactive metabolite in people, CBD-7-acid is the major metabolite of CBD and has a long half-life, ranging from 22 to 33 h in humans (FDA CDER, 2017, Taylor et al., 2018). In a study investigating the pharmacokinetics of CBD and its metabolites following CBD paste administration in horses, the authors reported the terminal half-life for CBD-7-acid was 79.85 h (Eichler et al., 2023). Fruge et al. (2025) observed the highest concentrations of CBD-7-acid at 72 h after the first of 14 daily doses; however, they did not sample past 72 h. The FDA CDER (2023) has stated an interest in CBD-7-acid, as it is considered a disproportionate metabolite in people. In the present study, samples were only collected intermittently up to 48 h after IH dosing, so an estimate of time to peak concentration or elimination half-life is impossible. We did observe detectable concentrations of CBD-7-acid in baseline samples from all six animals in round 3 and four of six animals in round 4. Animals receiving IH in round 3 had previously received IH in round 2, and animals receiving IH in round 4 had previously received IH in round 1. Although statistical difference was not analyzed,

the baseline CBD-7-acid concentrations from round 3 were higher than round 4. Cattle all underwent at least a 10-day washout period between rounds, suggesting that CBD-7-acid has a very prolonged half-life in cattle. Combined with data from Fruge et al. (2025), it appears that the elimination half-life of CBD-7-acid in cattle is long, as in people and horses.

In addition, to CBD-7-acid, we sporadically detected other cannabinoids in baseline samples. These other cannabinoids did not follow the temporal trend of the CBD-7-acid baseline samples. For CBC, CBG, CBG, 8-THC, and 9-THC, all positive samples came from one animal from round 1. The samples from this animal were analyzed at the beginning of a UPLC-MS/MS run following analysis of the calibration curve and a methanol wash. While it is possible that these large, neutral cannabinoids were not sufficiently removed from the column following the wash, resulting in false positive results, there were no cannabinoids detected in the methanol wash. The authors feel it is more likely that there were some early-eluting compounds or matrix interference producing these results. However, as previously mentioned, it is possible that individual animal variation in cannabinoid metabolism could explain the positive results from this one animal.

Limitations of this study include the small sample size and the cattle having extensive previous handling and human interaction. This prior exposure to people and manipulation during trials may have dampened some physiologic manifestations of stress measured in this study, including MNT, IRT, and cortisol values. Another limitation of this study is that the dosing regimen may not have allowed time for cannabinoids to reach effective concentrations during the transport period. In our previous report on the plasma pharmacokinetics of CBDA, we found time to maximum concentration was 11.8 h (Kleinhenz et al., 2020a). Pharmacodynamic studies evaluating effective cannabinoid concentrations for specific outcomes in cattle have not been

reported. While the dosing regimen chosen may have precluded some pharmacodynamic changes from occurring during the transport period itself, we observed complex effects of IH on blood glucose and prostaglandin concentrations. Interaction with transport and time effects makes interpretation of these results difficult. While the single dose can be viewed as a limitation, this regimen was chosen by the authors because it represents the most practical situation for producers, should further research (and approval of IH products for cattle) indicate that IH may have use as a therapeutic agent for alleviating negative effects of transport. Performing a study to evaluate these same outcomes under steady-state conditions would be enlightening, as one of the main goals of IH research in livestock is to pursue IH products as feed ingredients. Animals eating a diet containing IH would presumably be exposed for extended periods and would reach steady-state concentrations of cannabinoids.

As the IH market continues to grow, investigating potential therapeutic applications of IH and IH byproducts in cattle production will be pivotal. This study evaluated a wide range of outcome variables, which can serve as a basis for the development of future studies regarding transportation and associated stress in cattle. Based on our results and prior literature, we believe that PGEM and cortisol are the most promising avenues of future research regarding the use of IH as a potential therapeutic for transport stress in cattle.

Funding and disclaimer

Figure 4.1. Diagrammatic representation of treatment sequences and study design.[6]

Figure 4.2. Weight change (kg) (mean, 95% CI) over time for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH.[7]

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[7] Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Outcomes were assessed at -24, 0, 8, 24, 32, and 48 h relative to dosing and transport, with the 0 h weight used as the baseline value for calculation of weight change. Data points with different alphabetical identifiers are significantly different (p ≤ 0.05).

Figure 4.3. Step count data (mean, 95% CI) of Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[8]

[8] Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Accelerometric data were collected continuously throughout the study; data were collated into the following groups and converted into an hourly measurement: -24 to 0 h, 0 to 9 h, 9 to 24 h, and 24 to 48 h. Bars noted by different alphabetical identifiers are significantly different ($p \leq 0.05$).

Figure 4.4. White blood cell count (A), neutrophil count (B), blood glucose (C), and total protein (D) outcomes (mean, 95% CI) for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[9]

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[9] Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Outcomes were measured at -24, 8, and 48 h relative to dosing and transport. Bars noted by different alphabetical identifiers are significantly different ($p \leq 0.05$).

Figure 4.5. Cortisol AUC (A) and SAA (B) outcomes (mean, 95% CI) for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[10]

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[10] Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Cortisol AUC and SAA were log-transformed for analysis and backtransformed for presentation. Data noted by different alphabetical identifiers or asterisks are significantly different ($p \leq 0.05$).

Table 4.1. Cannabinoid content (dry-matter basis) of the IH (cultivar: Endurance HT) administered in this 4-phase block randomized Latin-square design study.[1]

Cannabinoid	Mean mg/g	Standard deviation mg/g	Limit of detection mg/g
THC	2.15	0.10	0.19
THCA	3.23	0.15	0.21
CBD	13.90	0.23	0.22
CBDA	79.88	2.64	0.28
CBC	1.85	0.13	0.09
CBCA	4.48	0.16	0.39
CBGA	2.06	0.03	0.52

[1]Holstein steers underwent repeat transport or control events and treatment with either a placebo or IH at a dose of 5.5 mg/kg CBDA given once by oral bolus immediately prior to the transport or control event.

Table 4.2. Weight (kg), MNT (kg*force; kgf), and IRT (°C) outcomes (mean; 95% CI) of Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[1]

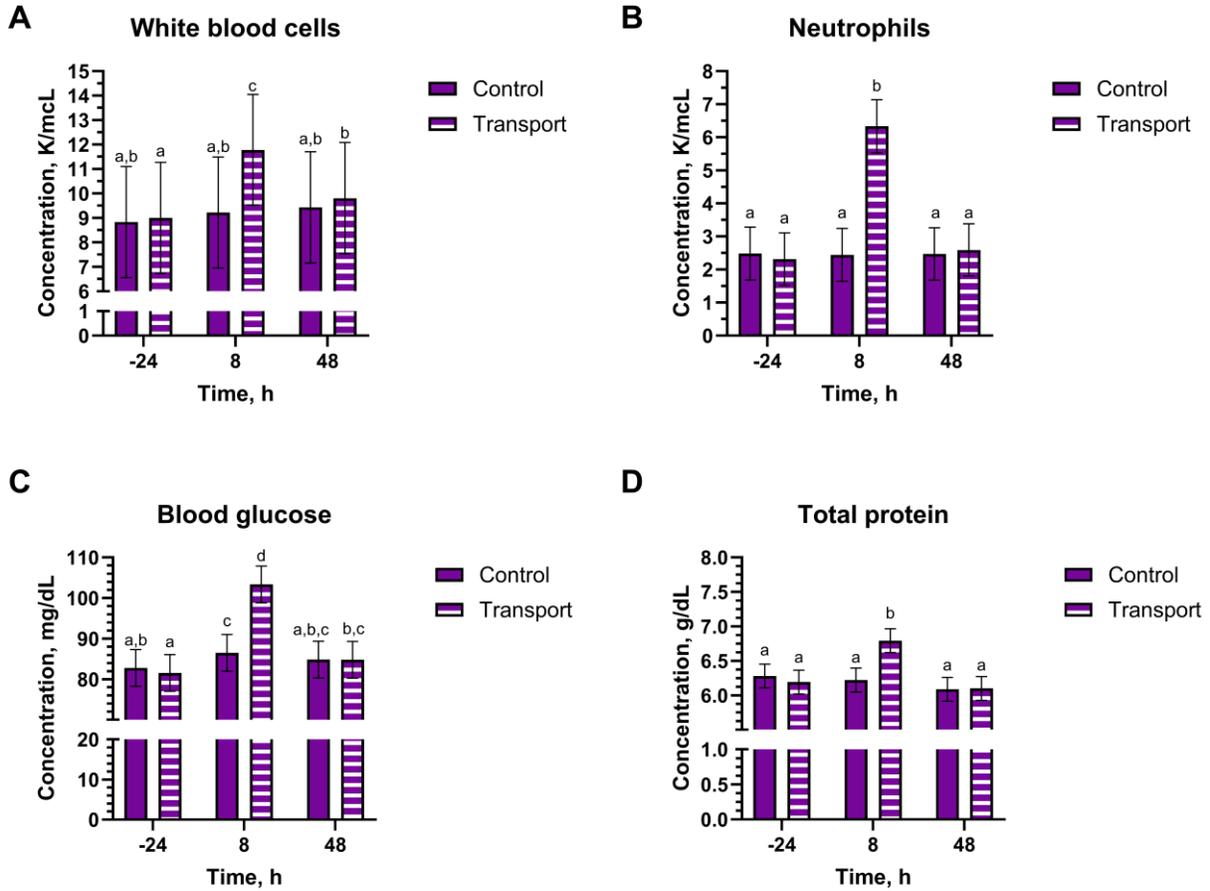| Parameter	Drug		Transport		P-value[2]						
Weight	HEMP	PLBO	TRANS	CNTL	Dr	Tr	Ti	Dr*Tr	Dr*Ti	Tr*Ti	Dr*Tr*Ti
Mean, kg	439 412 to 466	439 412 to 466	435 408 to 462	443 416 to 470	0.97	0.51	<0.0001	0.93	0.31	<0.0001	1.00
Change[3], kg	-6.9 -10.9 to -2.9	-9.1 -13.1 to -5.0	-13.0 -17.0 to -9.0	-2.9 -7.0 to 1.1	0.09	<0.0001	<0.0001	0.92	0.26	<0.0001	1.00
MNT											
LF mean, kgf	6.0 2.5 to 9.4	5.8 2.3 to 9.3	5.9 2.4 to 9.3	5.9 2.4 to 9.4	0.22	0.73	<0.0001	0.96	0.87	0.02	0.34
LH mean, kgf	5.0 2.3 to 7.7	4.9 2.3 to 7.6	4.9 2.2 to 7.5	5.1 2.4 to 7.8	0.54	0.08	<0.0001	0.70	0.16	0.08	0.48
LF change[3], %	24.7 -7.5 to 56.8	22.8 -9.4 to 54.9	26.7 -5.4 to 58.9	20.7 -11.4 to 52.8	0.81	0.44	<0.0001	0.43	0.84	0.06	0.62
LH change[3], %	24.9 -30.7 to 80.5	28.3 -27.3 83.9	25.5 -30.1 to 81.1	27.6 -28.0 to 83.2	0.64	0.77	<0.0001	0.51	0.40	0.17	0.72
IRT											
L eye max, °C	36.5 35.9 to 37.1	36.4 35.7 to 37.0	36.5 35.9 to 37.2	36.3 35.7 to 37.0	0.20	0.07	0.003	0.80	0.52	0.07	0.42
L hoof max, °C	20.3 14.4 to 26.2	19.9 14.0 to 25.8	20.0 14.1 to 25.9	20.2 14.3 to 26.1	0.53	0.80	0.009	0.71	0.13	0.47	0.23
L eye change[3], %	1.2 -0.8 to 3.3	0.9 -1.1 to 3.0	1.8 -0.3 to 3.8	0.4 -1.7 to 2.4	0.49	0.001	0.0004	0.008	0.47	0.30	0.86
L hoof change[3], %	40.7 -79.0 to 160.3	38.6 -81.0 to 158.2	37.4 -82.3 to 157.0	41.9 -77.8 to 161.5	0.76	0.51	<0.0001	0.34	0.20	0.93	0.69

[1]Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Outcomes were assessed at -24, 8, 24, 32, and 48 h relative to dosing and transport. Weight was additionally measured at 0 h.

[2]Drug – Dr; Transport – Tr; Time – Ti, [3]'Change' refers to change from baseline (0 h for weight, -24 h for MNT and IRT) values for that parameter

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Table 4.3. Accelerometric parameters (mean; 95% CI) of Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[1]
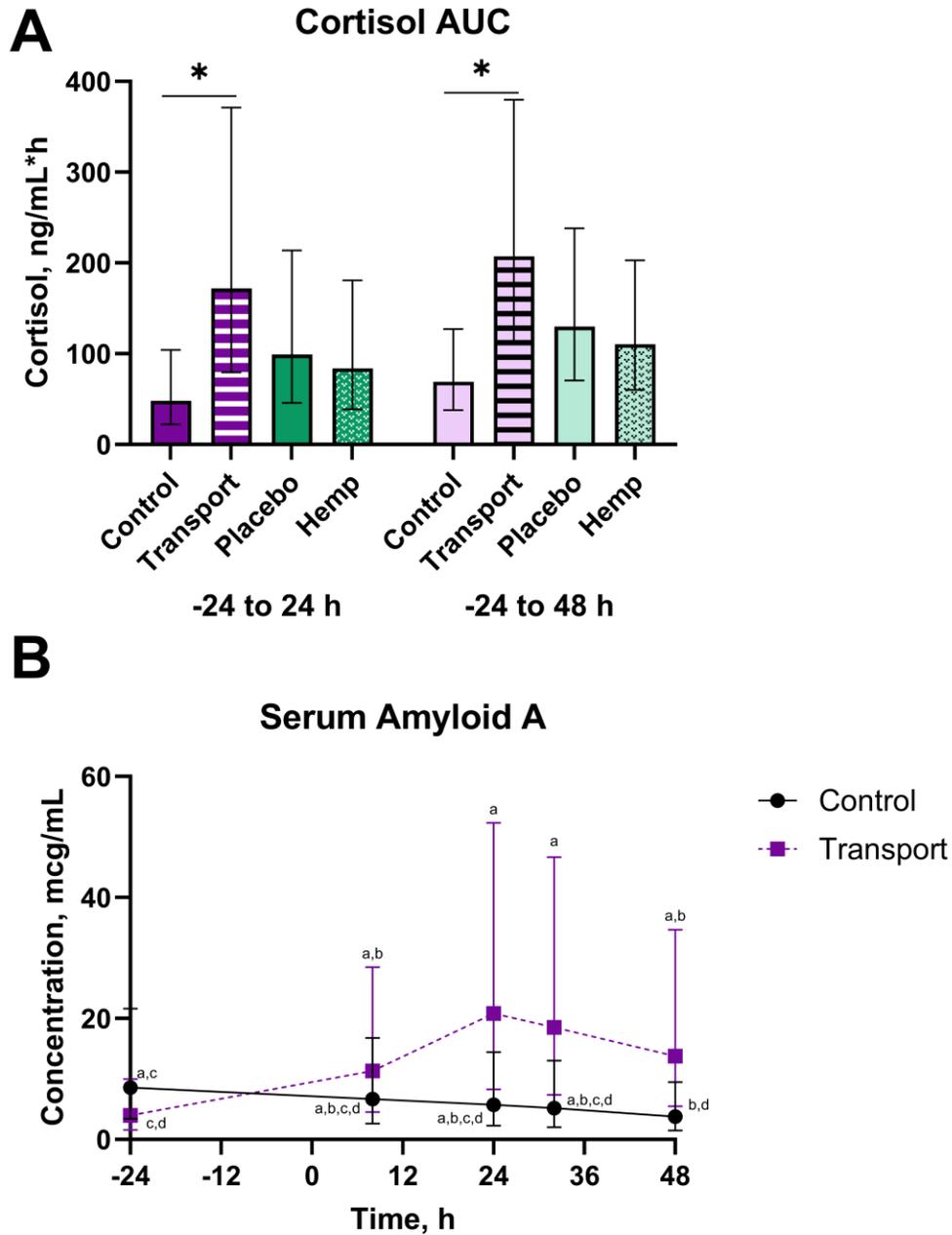
Parameter	Drug		Transport		P-value[2]						
	HEMP	PLBO	TRANS	CNTL	Dr	Tr	Ti	Dr*Tr	Dr*Ti	Tr*Ti	Dr*Tr*Ti
Motion index	528	531	602	457	0.89	<0.0001	<0.0001	0.32	0.76	<0.0001	0.87
	426 to 630	429 to 634	500 to 704	355 to 560							
Steps	120	121	137	104	0.83	<0.0001	<0.0001	0.39	0.81	<0.0001	0.82
	99 to 140	100 to 141	116 to 157	83 to 124							
Lying bouts	0.38	0.37	0.30	0.46	0.54	<0.0001	<0.0001	0.79	0.51	<0.0001	0.77
	0.31 to 0.46	0.30 to 0.45	0.23 to 0.37	0.38 to 0.53							
Standing ratio	0.51	0.51	0.57	0.45	0.84	<0.0001	<0.0001	0.46	0.94	<0.0001	0.65
	0.49 to 0.53	0.49 to 0.53	0.55 to 0.59	0.42 to 0.47							
Lying ratio	0.49	0.49	0.43	0.55	0.96	<0.0001	<0.0001	0.39	0.94	<0.0001	0.71
	0.47 to 0.51	0.47 to 0.51	0.40 to 0.45	0.53 to 0.58							

[1]Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Accelerometric data were collected continuously throughout the study; data were collated into the following groups and converted into an hourly measurement: -24 to 0 h, 0 to 9 h, 9 to 24 h, and 24 to 48 h.

[2]Drug – Dr; Transport – Tr; Time – Ti

Table 4.4. Kinetic gait analysis outcomes (mean; 95% CI) for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[1]

Parameter	Drug		Transport		P-value[2]						
	HEMP	PLBO	TRANS	CNTL	Dr	Tr	Ti	Dr*Tr	Dr*Ti	Tr*Ti	Dr*Tr*Ti
Front gait distance, cm	149 137 to 160	147 135 to 159	146 134 to 157	150 138 to 162	0.81	0.37	0.006	0.53	0.95	0.92	0.24
Front gait velocity, cm/s	120 107 to 133	125 113 to 138	123 110 to 136	123 110 to 136	0.45	0.97	0.003	1.00	0.95	0.18	0.29
Front stance, s	0.88 0.71 to 1.04	0.83 0.67 to 1.00	0.87 0.71 to 1.03	0.84 0.68 to 1.00	0.35	0.49	0.16	0.84	0.67	0.47	0.49
Front stride, cm	145 135 to 155	148 138 to 157	147 137 to 157	145 136 to 155	0.51	0.58	<0.0001	0.81	0.95	0.93	0.90
Front force, kg	147 143 to 151	142 137 to 146	146 141 to 150	143 139 to 148	0.22	0.52	<0.0001	0.89	0.90	0.73	0.57
*Front FTI, kg*s*	87.3 82.9 to 91.6	80.7 76.3 to 85.0	84.6 80.2 to 88.9	83.4 79.0 to 87.7	0.11	0.77	<0.0001	0.94	0.92	0.39	0.26
Front pressure, kg/cm²	4.1 3.6 to 4.6	4.0 3.6 to 4.5	4.1 3.6 to 4.6	4.1 3.6 to 4.6	0.12	0.43	0.054	0.39	0.67	0.31	1.00
Rear stance, s	0.84 0.78 to 0.90	0.81 0.75 to 0.87	0.82 0.76 to 0.88	0.83 0.77 to 0.89	0.46	0.73	0.25	0.90	0.92	0.38	0.47
Rear stride, cm	146 136 to 157	145 135 to 156	147 136 to 157	145 134 to 155	0.75	0.54	<0.0001	0.74	0.90	1.00	0.43
Rear force, kg	132 120 to 145	133 120 to 145	130 117 to 143	135 122 to 148	0.97	0.26	<0.0001	0.68	0.76	0.30	0.66
*Rear FTI, kg*s*	76.7 65.0 to 88.4	72.7 61.1 to 84.4	74.3 62.6 to 85.9	75.2 63.5 to 86.9	0.42	0.85	0.0003	0.56	0.85	0.08	0.52
Rear pressure, kg/cm²	3.9 3.4 to 4.4	3.9 3.4 to 4.5	3.9 3.4 to 4.4	3.9 3.4 to 4.5	0.95	0.67	0.009	0.04	0.11	0.40	0.93

[1] Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Outcomes were measured at -24, 8, 24, 32, and 48 h relative to dosing and transport.

[2] Drug – Dr; Transport – Tr; Time – Ti

Table 4.5. Select parameters (mean; 95% CI) from complete blood count and serum biochemistry analyses for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[1]

Parameter	RI[3]	Drug		Transport		P-value[1]						
		HEMP	PLBO	TRANS	CNTL	Dr	Tr	Ti	Dr*Tr	Dr*Ti	Tr*Ti	Dr*Tr*Ti
WBC[4], K/μL	4.9 – 12.0	9.8 / 7.5 to 12.1	9.6 / 7.3 to 11.9	10.2 / 7.9 to 12.5	9.2 / 6.9 to 11.5	0.61	0.01	<0.0001	0.84	0.42	<0.0001	0.81
NEU[5], K/μL	1.8 – 6.3	3.1 / 2.3 to 3.9	3.1 / 2.3 to 3.9	3.7 / 3.0 to 4.5	2.5 / 1.7 to 3.2	0.90	<0.0001	<0.0001	0.82	0.27	<0.0001	0.88
MONO[6], K/μL	0.0 – 0.8	0.6 / 0.4 to 0.8	0.6 / 0.4 to 0.8	0.6 / 0.4 to 0.8	0.6 / 0.4 to 0.8	0.93	0.75	0.01	0.86	0.96	0.04	0.53
LYMPH[7], K/μL	1.6 – 5.6	5.7 / 3.9 to 7.6	5.6 / 3.7 to 7.5	5.6 / 3.7 to 7.4	5.8 / 3.9 to 7.7	0.68	0.51	<0.0001	0.80	0.13	<0.0001	0.46
Fibrinogen, mg/dL	300 – 700	388 / 323 to 452	402 / 338 to 466	388 / 324 to 453	401 / 337 to 465	0.59	0.64	0.42	0.10	0.38	0.33	0.55
HCT[8], %	22 – 33	29 / 28 to 31	29 / 28 to 31	30 / 28 to 31	29 / 28 to 31	0.76	0.15	<0.0001	0.052	0.53	0.002	0.77
Glucose, mg/dL	29 – 73	87 / 82 to 91	88 / 83 to 93	90 / 85 to 95	85 / 80 to 89	0.26	<0.0001	<0.0001	0.30	0.68	<0.0001	0.03
Total protein, g/dL	6.0 – 9.0	6.3 / 6.1 to 6.4	6.3 / 6.1 to 6.5	6.4 / 6.2 to 6.5	6.2 / 6.0 to 6.4	0.84	0.03	<0.0001	0.32	0.90	<0.0001	0.13

[1]Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Outcomes were measured at -24, 8, and 48 h relative to dosing and transport.

[2]Drug – Dr; Transport – Tr; Time – Ti [3]Reference interval [4]White blood cell concentration – WBC [5]Segmented neutrophil concentration – NEU [6]Monocyte concentration – MONO [7]Lymphocyte concentration – LYMPH [8]Hematocrit (calculated) – HCT

Table 4.6. Cortisol, PGEM, and SAA outcomes (mean; 95% CI) for Holstein steers undergoing repeat transport or control events and treatment with either a placebo or IH in a 4-phase block randomized Latin-square design study.[1]

Parameter	Drug		Transport		P-value[2]						
Cortisol	HEMP	PLBO	TRANS	CNTL	Dr	Tr	Ti	Dr*Tr	Dr*Ti	Tr*Ti	Dr*Tr*Ti
Average, ng/mL	1.3 0.8 to 2.1	1.0 0.6 to 1.7	1.6 0.9 to 2.6	0.8 0.5 to 1.4	0.26	0.002	<0.0001	0.37	0.52	0.15	0.66
AUC (-24 – 24 h), ng/mL*h	84 39 to 181	99 46 to 214	172 80 to 371	48 22 to 104	0.51	<0.0001	N/A	0.81	N/A	N/A	N/A
AUC (-24 – 48 h), ng/mL*h	111 60 to 203	130 71 to 238	207 113 to 380	69 38 to 127	0.49	<0.0001	N/A	0.79	N/A	N/A	N/A
PGEM											
Average, pg/mL	18.7 16.0 to 21.8	16.8 14.4 to 19.6	17.3 14.9 to 20.2	18.1 15.6 to 21.1	0.17	0.55	0.02	0.76	0.17	0.62	0.11
Change, %	-4.5 -22.6 to 13.5	5.3 -12.7 to 23.3	-2.5 -20.5 to 15.5	3.3 -14.8 to 21.3	0.10	0.32	0.04	0.52	0.23	0.85	0.03
SAA, µg/mL	7.8 3.1 to 19.4	8.8 3.5 to 22.1	11.9 4.8 to 29.7	5.8 2.3 to 14.4	0.75	0.07	0.0001	0.34	0.89	<0.0001	0.20

[1]Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event. Average cortisol, cortisol AUC -24 to 24 h, cortisol AUC -24 to 48 h, average PGEM, and average SAA were log-transformed for analysis. Means and 95% CI are presented as back-transformed values.

[2]Drug – Dr; Transport – Tr; Time – Ti

Table 4.7. Inter-day accuracy of QC samples and LLOQ for cannabinoids analyzed via UPLC-MS

		Accuracy		
	LLOQ	4.75 ng/mL QC	47.5 ng/mL QC	95 ng/mL QC
Cannabinoid	*ng/mL*	*%*	*%*	*%*
CBC	1.0	104.0	101.1	98.3
CBD-6-OH	2.5	86.9	94.4	101.3
CBD-7-acid	1.0	100.0	96.2	90.0
CBD-7-OH	2.5	93.7	99.4	98.8
CBD	1.0	101.3	101.1	98.1
CBDA	2.5	88.4	99.3	100.9
CBDV	2.5	89.1	89.8	88.8
CBDVA	2.5	100.9	109.3	99.2
CBG	1.0	104.9	102.0	97.8
CBGA	2.5	91.2	95.6	94.6
CBN	1.0	104.8	102.9	101.3
8-THC	1.0	104.3	101.3	99.4
9-THC	1.0	104.5	100.3	98.0
THC-11-OH	1.0	103.4	100.6	99.7
THC-acid	2.5	106.6	104.2	99.1
THC-acid-glu	1.0	100.9	102.9	102.0
THCA	1.0	104.2	103.2	100.5
THCV	1.0	91.1	96.4	95.8

Table 4.8. Cannabinoid concentrations (median; 95% CI) for Holstein steers undergoing repeat transport or control events receiving IH in a 4-phase block randomized Latin-square design study.[1]

	Time, h									
Cannabinoid	**-24**		**8**		**24**		**32**		**48**	
Round 1	*Med*	*CI*	*Med*	*CI*	*Med*	*CI*	*Med*	*CI*	*Med*	*CI*
CBC	0	0, 9.8	N/D	N/D	N/D	N/D	0	0, 2.7	N/D	N/D
CBD-6-OH	N/D	N/D	N/D	N/D	0	0, 2.5	N/D	N/D	0	0, 2.3
CBD-7-acid	0	0, 1.8	1.0	0, 2.3	17.5	8.1, 28.5	20.1	10.4, 35.8	34.2	15.2, 56.5
CBD-7-OH	N/D	N/D	N/D	N/D	0	0, 3.3	N/D	N/D	0	0, 2.9
CBDA	N/D	N/D	55.8	0, 184.6	97.8	75.7, 143.8	117.5	64.9, 144.9	59.3	23.1, 103.9
CBDVA	0	0, 2.3	5.4	0, 11.8	5.2	1.5, 13.2	3.6	0.3, 9.0	0	0, 3.4
CBG	0	0, 10.3	0	0, 9.7	0	0, 3.0	N/D	N/D	N/D	N/D
CBGA	N/D	N/D	0	0, 8.3	N/D	N/D	N/D	N/D	0	0, 2.3
CBN	0	0, 13.0	0	0, 15.4	0	0, 5.4	0	0, 2.3	0	0, 4.9
9-THC	0	0, 9.1	0	0, 7.7	N/D	N/D	N/D	N/D	N/D	N/D
8-THC	N/D	N/D	0	0, 8.4	0	0, 2.4	N/D	N/D	0	0, 2.4
THC-acid	N/D	N/D	N/D	N/D	0	0, 0.8	N/D	N/D	0	0, 2.1
THC-11-OH	N/D	N/D	N/D	N/D	0	0, 0.8	N/D	N/D	0	0, 1.2
THCA	0	0, 3.1	3.5	1.1, 6.3	8.1	6.4, 9.7	8.5	6.1, 11.3	5.9	1.9, 7.7
Round 2										
CBD-7-acid	0	0, 1.1	2.0	0.4, 3.5	26.9	17.9, 35.9	23.1	12.8, 38.6	42.5	34.6, 50.2
CBDA	0	0, 11.9	84.2	47.0, 125.4	85.0	63.1, 109.4	95.2	70.7, 150.5	40.6	32.3, 49.8
CBDVA	N/D	N/D	6.0	1.9, 10.0	5.0	1.4, 9.4	3.8	0.1, 7.3	0	0, 2.4
THCA	0	0, 4.4	3.4	0.9, 4.7	6.2	4.5, 7.6	6.7	4.6, 9.0	4.0	2.8, 5.3
Round 3										
CBD-7-acid	13.9	10.7, 18.3	13.2	9.2, 20.0	45.1	30.4, 61.9	29.4	21.7, 49.9	70.1	37.2, 96.3
CBDA	0	0, 5.4	59.9	41.6, 85.9	127.1	110.8, 179.4	136.2	100.0, 189.4	62.7	39.0, 93.7
CBDVA	N/D	N/D	5.2	1.7, 7.2	4.9	0.8, 11.3	9.5	1.0, 13.6	0	0, 3.1
CBGA	N/D	N/D	N/D	N/D	N/D	N/D	0	0, 1.9	N/D	N/D
THCA	N/D	N/D	2.9	1.8, 4.3	8.0	5.1, 10.4	8.1	5.1, 11.3	5.1	3.4, 6.8
Round 4										
CBD-7-acid	1.8	0.2, 2.6	2.2	0.7, 3.8	20.7	11.0, 32.9	22.5	14.5, 32.6	36.3	17.6, 64.8
CBDA	0	0, 26.9	58.4	27.3, 98.4	74.5	48.0, 142.1	69.8	30.0, 132.9	44.1	14.0, 78.9
CBDVA	N/D	N/D	6.1	3.1, 11.7	5.2	1.3, 11.6	5.5	1.5, 9.7	0	0, 3.8
CBGA	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0	0, 2.5
THCA	0	0, 2.6	3.5	0, 14.5	6.2	4.8, 8.1	6.4	4.8, 8.0	5.7	4.1, 8.0

[1]Industrial hemp was dosed at 5.5 mg/kg CBDA and given once by oral bolus immediately prior to the transport or control event (0 h)

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Chapter 5 - Plasma cannabinoid concentrations and transference during long-term industrial hemp administration in cattle[11]

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ABSTRACT

With recent legalization of industrial hemp (IH) production and increased interest in including IH and its byproducts in cattle feed, there is a need to establish the pharmacokinetic profiles of cannabinoids in cattle and guidelines for animal surveillance programs to ensure safety of animal products entering the food supply. Our group has previously described the pharmacokinetics of cannabidiolic acid (CBDA) and concentrations of other cannabinoids in plasma. In the present study, the plasma cannabinoid concentrations in twelve (12) Holstein steers receiving alfalfa pellet placebo, chlortetracycline (CTC) pellets (1.1 mg/kg/d), IH (5.5 mg/kg/d CBDA; HEMP), or a combination of CTC and IH (COMBO) once daily for 63 days were evaluated. Plasma samples were collected every 7 days from day -7 to 77. Twelve cannabinoids were detected above the limit of quantification (LOQ), with the cannabinoid (−)-7-nor-7-carboxy cannabidiol (CBD-7-acid) reaching the highest concentrations. All cannabinoids except (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-acid-glu) and CBD-7-acid were below LLOQ by 14 days after final hemp administration. In cattle not receiving IH, CBD-7-acid and CBDA were detected in multiple animals and timepoints. The cannabinoid THC-acid-glu (an inactive metabolite of the psychoactive cannabinoid Δ9-tetrahydrocannabinol (9-THC)) was detected after final IH administration in all groups. Both 9-THC and its precursor, THCA, were also detected above LOQ in a singular sample from an animal in the CTC group. These findings suggest that cattle not receiving IH could have detectable concentrations of cannabinoids in the blood if cohoused with animals that are exposed to IH. Based on our results, CBD-7-acid may be a useful tool for screening cattle for IH exposure. However, given the positive results in groups not administered IH, a confirmatory test or specific criteria for interpreting CBD-7-acid concentrations would be warranted. These data will help inform decisions regarding surveillance

and tolerances for cannabinoid testing in food animals and animal products entering the food supply.

Key words: cannabinoids, cattle, exposure, industrial hemp, plasma, transference

Abbreviations

8-THC – Δ8-tetrahydrocannabinol

9-THC – Δ9-tetrahydrocannabinol

CBC – cannabichromene

CBCA – cannabichromenic acid

CBD – cannabidiol (CBD)

CBD-7-acid – (−)-7-nor-7-carboxy cannabidiol

CBDA – cannabidiolic acid

CBDV – cannabidivarin

CBDVA – cannabidivarinic acid

CBG – cannabigerol

CBGA – cannabigerolic acid

CBLA – cannabicyclolic acid

CBN – cannabinol

FDA – United States Food and Drug Administration

IACUC – institutional animal care and use committee

IH – industrial hemp

LLOQ – lower limit of quantification

THC-11-OH – (±)-11-hydroxy-Δ9-tetrahydrocannabinol

THC-acid – (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol

THC-acid-glu – (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide

THCA – Δ9-tetrahydrocannabinolic acid A

THCV – Δ9-tetrahydrocannabivarin

THCVA – Δ9-tetrahydrocannabivarinic acid.

UPLC-MS – ultra-performance liquid chromatography triple quadrupole mass spectrometry

USDA – United States Department of Agriculture

INTRODUCTION

Commercial cultivation of industrial hemp (IH; *Cannabis sativa* with ≤ 0.3% Δ9-tetrahydrocannabinol) was legalized with the 2018 Farm Bill, opening the door for a variety of new markets (Johnson, 2021). The entire IH plant has been leveraged for various products, including fiber, seeds and seed products, and cannabinoid oil extracts (Kleinhenz et al., 2020b). In particular, cannabidiol (CBD) oil extract is a major retail interest in the United States. After extracting CBD (or other cannabinoids) from the cannabinoid-rich flowers and leaves, a large amount of plant material is left behind as "waste". Because ruminant species, including cattle, are able to digest fibrous plant material, there is increased interest in including IH and its various byproducts in cattle feed. Our group has demonstrated that IH has a favorable digestibility profile and previous work has indicated that various IH products are safe for inclusion in cattle diets (Kleinhenz et al., 2020b, Addo et al., 2023, Smith et al., 2023, Wang et al., 2023, Irawan et al., 2024, Fruge et al., 2025, Irawan et al., 2025).

Due to concerns in exposed cattle regarding transfer of cannabinoids into edible products, such as meat or milk, there is a need to establish the pharmacokinetics and tissue depletion profile of various cannabinoids. Our group has previously reported plasma pharmacokinetics and behavioral changes following single and multiple IH doses (Kleinhenz et al., 2020a, Kleinhenz et

al., 2022). Other groups have published on the depletion of cannabinoids in various tissues (Smith et al., 2023, Fruge et al., 2025, Irawan et al., 2025).

As regulatory agencies investigate potential approval of IH as a feed ingredient, there is a critical need to establish tests for identifying exposed cattle entering the food supply. While methods for tissue matrices are important, analysis of plasma would enable detection of exposed cattle prior to slaughter. Antemortem diagnosis of IH exposure would reduce animal waste by identifying concerning concentrations of cannabinoid exposure prior to slaughter, allowing producers the time to address any potential violative residues. Currently, there is little research on the long-term plasma depletion of cannabinoids after withdrawal from IH administration, with a single paper evaluating plasma cannabinoid concentrations up to 8 days after withdrawal (Smith et al., 2023). Establishing the time frame for depletion and determining the ideal cannabinoid for use as a surrogate for exposure will assist in regulatory planning of surveillance measures.

The objective of this study was to determine the plasma cannabinoid concentrations in cattle during and after prolonged administration of IH flowers.

MATERIALS AND METHODS

Ethics statement and animal disposition

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4749). All study activities were conducted in conformity to requirements from the United States Department of Agriculture, the State of Kansas, and American Association for Accreditation of Laboratory Animal Care according to *The Guide for the Care and Use of Agricultural Animals in Research and Teaching (ADSA ASAS PSA, 2020)*. Six of the animals (receiving either placebo or CTC) were sold to a local sale barn

following study conclusion; the remaining six animals, which had previously received an experimental vaccine, were humanely euthanized via captive bolt and intravenous administration of supersaturated magnesium sulfate in accordance with AVMA guidelines.

Animals and Housing

Twelve (*n* = 12) Holstein steers 2 yr of age and weighing (± SD) 644 ±145 kg (1416 ± 319 pounds) were enrolled. Animals had been previously acclimated to the research facility and were group-housed in an outdoor dirt pen with access to shelter throughout the study period. Animals were fed a custom grain mix twice daily and had ad libitum access to grass hay and water via an automated watering device during the entire study. The pen area supplied per calf exceeded the guidelines established in the Guide for the Care and Use of Agricultural Animals in Research and Teaching.

All animals had previously been used in a trial investigating an experimental *Anaplasma marginale* vaccine (IACUC #4643); 6 of the animals received the vaccine, and the remaining 6 were unvaccinated controls. All 12 animals were experimentally infected with *A. marginale* str. St. Maries during this previous trial and were confirmed positive on RT-qPCR within 30 d prior to initiation of the present study. Due to the biosecurity risks with *A. marginale*, steers were housed in a pen that did not share a fence-line or direct contact with other animals and grass and weeds were mowed within 10 feet of the experimental pens. All steers were treated with acaricide and fly control (UltraBoss Pour-On Insecticide, Merck Animal Health, Rahway, NJ 07065) every 2 wk,

Experimental design

This study was intended to be a pilot study investigating the effects of IH on *A. marginale* cELISA and RT-qPCR parameters. All animals were confirmed to be cELISA and

RT-qPCR positive for *A. marginale* str. St. Maries within 30 d of initiation of this study. However, analysis of samples from day 0 of this study revealed multiple animals with negative results on both tests. Samples had been frozen and stored for batch analysis, thus, the negative results were not observed until well into the study. In light of this unforeseen complication, the authors elected to report the plasma cannabinoid data, as this has not been reported previously.

A restricted randomization technique was applied in order to minimize the number of animals that would have to be euthanized (as animals receiving hemp or the experimental vaccine could not enter the food chain). The 6 experimentally-vaccinated animals were randomized to receive one of the hemp-containing treatments and the 6 animals unvaccinated animals were randomized to receive one of the treatments without IH. There were 3 animals in each treatment. Treatments included daily administration of (1) alfalfa pellet placebo (PLBO), (2) chlortetracycline pellets (1.1 mg/kg/d; Aureomycin, Zoetis, Parsippany, NJ 07054; CTC), (3) IH (5 mg/kg/d CBDA; HEMP), or (4) combination treatment with CTC and IH as described above (COMBO) for 63 d. The obvious nature of the treatments precluded blinding of the investigators administering treatments.

Treatment preparation and administration

Prior to study initiation, the CBDA content as a percentage of total IH weight was determined using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS) and was used to calculate IH doses on an as-fed basis. Cattle in the hemp groups received IH at a dose of 5 mg/kg cannabidiolic acid (CBDA) by oral bolus. Hemp flower material was finely chopped using a food chopper. Ground IH was placed in gelatin capsules and the weight of the capsules (with empty capsule weight tared) was recorded. Chlortetracycline dosages were calculate weekly based on the updated steer weights and doses were weighed and

placed into pre-labeled bags. Aliquots of alfalfa pellets similar to the average weight of chlortetracycline pellets were placed into labeled bags.

Industrial hemp was grown and handled in keeping with licensing requirements under the Kansas Department of Agriculture Industrial Hemp Research Program (license numbers: KDA-0621466839 and KDA-0302873296). The cultivars Endurance HT and CW117 were used (harvested October 2021).

Feed tubs were labeled and assigned to one steer for the entire study to prevent any cross-contamination of treatments. Steers in the PLBO and CTC groups received their treatments loose in their feed tubs with approximately 500 g of sweet feed. Steers in the HEMP or COMBO groups were restrained in a chute, a rope halter placed, and the IH administered via oral bolus; they were then released from the chute and given approximately 500 g of sweet feed in their feed tubs. Steers were monitored during feeding to ensure CTC crumbles and IH boluses were consumed. Occasionally, some animals had to receive part of their CTC crumbles in a bolus or the IH capsules re-administered if not all of the crumbles were consumed or if the boluses were spit out.

Sample collection

Whole blood samples were collected at -7, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 d after initial treatment for complete blood count analysis and cannabinoid concentrations. The 0 d timepoint was collected immediately prior to the first treatment administration. Jugular or coccygeal venipuncture were performed with an 18-gauge 1.5-inch needle and 20 mL syringe or vacutainer system. The blood samples were directly placed into 2 pre-labeled 6 mL K3-EDTA blood tubes, placed on ice, and transferred to the lab. Blood for complete blood count analysis was immediately analyzed using an IDEXX ProCyte Dx hematology analyzer (IDEXX

Laboratories, Inc., Westbrook, Maine 04092) and a manual packed cell volume was calculated. Blood smear analysis was performed on any samples exhibiting reticulocytosis or band cells. Blood for cannabinoid analysis was centrifuged at 3,500 relative centrifugal force for 10 min and the plasma was aliquoted into cryovials and frozen at -80°C for future analysis.

Animal monitoring

Due to potential for recrudescence of clinical signs due to anaplasmosis, animals underwent a weekly monitoring protocol to screen for signs of disease. This screening included a complete blood count, manual hematocrit, rectal temperature, and observation of behavior. If the red blood cell count or manual hematocrit were below the reference interval, rectal temperature was > 105°F, or inappetence, standing with legs stretched out, excessive recumbency, or reluctance to rise were observed, the animals were evaluated by a veterinarian and a treatment plan was determined by discussion between study personnel, the veterinarian, and the IACUC attending veterinarian. Animals were also observed daily during treatment administration and any signs of disease reported to the principal investigator and IACUC attending veterinarian.

Blood cannabinoid concentrations

Plasma cannabinoids were measured as previously described (Kleinhenz et al., 2020a). Briefly, all solvents used such as methanol, acetonitrile, isopropanol, and formic acid were LC–MS grade. Individual cannabinoid standards were purchased as solutions in methanol (Cerilliant Corporation, Round Rock, TX), including: (+)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide (THC-acid-glu), (−)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-acid), (±)-11-hydroxy-Δ9-tetrahydrocannabinol (THC-11-OH), cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), cannabidiol (CBD), cannabidiolic acid (CBDA), Δ9-tetrahydrocannabinolic acid A (THCA), cannabigerolic acid (CBGA), cannabigerol (CBG), Δ9-

tetrahydrocannabinol (9-THC), Δ8-tetrahydrocannabinol (8-THC), cannabichromene (CBC), Δ9-tetrahydrocannabivarin (THCV), cannabichromenic acid (CBCA), cannabinol (CBN), (-)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-acid). Cannabinoid analogs used as internal standards included (±)-cis-11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide-d_3 (THC-acid-glu-d_3), cannabidiol-d_3 (CBD-d_3), Δ9-tetrahydrocannabinol-d_3 (9-THC-d3), (±)-11-nor-9-carboxy-Δ9-tetrahydrocannabinol-d_9 (THC-acid-d_9), (±)-11-hydroxy-Δ9-tetrahydrocannabinol-d_3 (THC-11-OH-d_3), and cannabichromene-d_9 (CBC-d_9). All cannabinoids standards were stored at $-20°C$.

On the day of analysis, plasma samples were thawed at room temperature. Plasma, internal standard mixture (200 ng/mL), and acetonitrile with 0.1% formic acid were combined to precipitate plasma proteins. Internal standard mixture was not added to the negative controls. Following vortexing and centrifugation, the supernatant was diluted with ultra-pure 18 Ω water. Samples were loaded onto a solid phase extraction plate via a nitrogen positive pressure manifold. Washes were performed with methanol:water (25:75) and elution was performed with acetonitrile:methanol (90:10). Eluates were diluted with water prior to analysis.

Cannabinoid analysis was performed using an Acquity H class UPLC and a TQ-S triple quadrupole mass spectrometer (Waters Corp., Milford, MA). Chromatographic separation was achieved using a UPLC column (100 × 2.1 mm, 1.8 μ, Eclipse Plus C18, Agilent Technologies, Santa Clara, CA) heated at 55°C. The mobile phase consisted of a gradient of water containing 0.1% formic acid (A) and acetonitrile (B) as follows: 0 min: 60% B, 6.50 min: 86% B, 7.50–9 min: 100% B, 9.01-12 min: 60% B. The flow rate was set at 0.5 mL/min, injection volume was 5 μL, and the run time per sample was 12 min. Data acquisition was performed using electrospray ionization in positive and negative mode using multiple reaction monitoring. Linear regression with a weighting factor of 1/X was used and accepted if the coefficient of correlation

R^2 was > 0.99. Calibration curves were linear from 0.1 to 100 ng/mL for all cannabinoids. The lower limit of quantification (LLOQ) for each detected cannabinoid is presented in Table 5.1.

Statistics

Cannabinoid concentrations were summarized and analyzed using statistical software (JMP Pro Version 16.0, SAS Institute Inc., Cary, NC, USA). Summary statistics were generated for each cannabinoid within each group and timepoint. Due to the small sample sizes, nonparametric analysis was performed. To analyze the effect of treatment (HEMP vs. COMBO) on cannabinoid concentrations, the Wilcoxon exact test was used. For analysis of time effects, the Friedman rank test was performed. Significance was set a priori at $P \leq 0.05$. Figures were made using GraphPad (GraphPad Prism, 10.0, La Jolla, CA, USA).

RESULTS

One animal had transient neutropenia on day 14 (resolved on recheck complete blood count the following day) and one animal was noted to be mildly lethargic on day 21. No other adverse events were noted during the study.

Mean cannabinoid concentrations of the IH cultivar Endurance HT were: 2.15 mg/g 9-THC, 3.23 mg/g THCA, 1.85 mg/g CBC, 4.48 mg/g CBCA, 13.90 mg/g CBD, 79.88 mg/g CBDA, and 2.06 mg/g CBGA. Mean cannabinoid concentrations of the IH cultivar CW117 were: 1.73 mg/g 9-THC, 3.40 mg/g THCA, 1.77 mg/g CBC, 5.05 mg/g CBCA, 10.39 mg/g CBD, 87.92 mg/g CBDA, 2.82 mg/g CBGA, and 2.68 mg/g CBDVA.

No cannabinoids were detected above the LLOQ on days -7 or 0 in any group. The cannabinoids CBCA, CBD-7-acid, CBD, CBDA, CBDVA, CBGA, CBLA, 9-THC, THCA, THCV, THCVA, and THC-acid-glu were detected above the LLOQ in at least one animal from one group. There were 3 THCV-positive samples (2 from the HEMP group, 1 from the COMBO

group) and 1 CBGA-positive sample (HEMP group). In the HEMP group, only CBD-7-acid was detectable above LLOQ on day 70 and both CBD-7-acid and THC-acid-glu were detectable above LLOQ on day 77. The COMBO group followed a similar trend, except that one animal also had a positive THC-acid-glu sample on day 70. Summary statistics for each cannabinoid within the HEMP and COMBO treatments at each timepoint are presented in Table 5.1. Concentrations of CBD-7-acid, CBDA, CBDVA, and THCA over time are presented in Fig. 5.1. Time did not have a significant effect on concentration for any cannabinoid ($P > 0.05$). There was a significant effect of treatment for CBD-7-acid concentrations ($P = 0.02$, two-tailed), where the COMBO group had higher rank concentrations than HEMP. There was no significant effect of treatment for any other cannabinoid ($P > 0.05$).

In the PLBO group, CBD-7-acid was detected above LLOQ at all timepoints except days -7, 0, and 7 [median: 0 ng/mL (95% CI 0.63 to 4.40); minimum: 0 ng/mL, maximum 32.2 ng/mL]. In the CTC group, CBD-7-acid was detected above LLOQ at all timepoints except days -7, 0, 7, 28, and 35 [median: 0 ng/mL (95% CI 0.31 to 1.24); minimum: 0 ng/mL, maximum: 6.8 ng/mL]. There were a total of 2 CBDA-positive samples each from the CTC (day 56 and 63) and PLBO (day 56) groups. The psychoactive cannabinoid, 9-THC, and its precursor, THCA, were detected in a single steer in the CTC group on day 70 (9-THC: 1.3 ng/mL, THCA: 29 ng/mL). In the CTC and PLBO groups, CBD-7-acid and THC-acid-glu were detectable above LLOQ on days 70 and 77 (CBD-7-acid range: 1.2 to 3.2 ng/mL; THC-acid-glu range: 1.4 to 5.3 ng/mL).

DISCUSSION

This report is the longest survey of plasma cannabinoids after withdrawal from IH administration, with samples collected 14 days after the last IH dose. A previous study by Smith et al. (2023) evaluating depletion of cannabinoids sampled plasma up to 8 days following the last

day of feeding hempseed meal. We detected a total of 12 cannabinoids, including 8 acidic cannabinoids. No cannabinoids were detected on days -7 or 0 in any group. We observed detectable concentrations of the cannabinoids CBD-7-acid and THC-acid-glu 14 days after the final dose of IH was administered, whereas other cannabinoids were not detected by 7 days after the final dose of IH. We did not observe a significant effect of time on cannabinoid concentrations. There was only a significant effect of treatment (HEMP vs. COMBO) for CBD-7-acid. In general, cannabinoid concentrations were relatively static throughout the feeding period, with consistently, numerically lower concentrations on day 28. A handful of cannabinoids were sporadically detected in cattle not receiving IH.

The cannabinoid signatures we described in this study are similar to previous reports that observed predominantly acidic cannabinoids in plasma (Kleinhenz et al., 2020a, Kleinhenz et al., 2022, Smith et al., 2023, Fruge et al., 2025). Studies evaluation IH flowers and leaves have detected CBCA, CBD-7-acid, CBD, CBDA, CBDVA, CBGA, THCA, and THCVA (Kleinhenz et al., 2020a, Kleinhenz et al., 2022, Fruge et al., 2025). In cattle receiving hempseed cake, Smith et al. (2023) reported detectable concentrations of CBCA, CBDA/THCA, CBDVA, and CBNA. Irawan et al. (2025) detected additional, non-acidic cannabinoids, including CBG, 9-THC, and THC-11-OH in cows fed spent hemp biomass (SHB). Compared to these studies, Wagner et al. (2022) detected mostly neutral cannabinoids, including CBD, CBDV, 9-THC, and THCV, along with THCA, in plasma of cows fed IH silage. Summarizing this previous literature, acidic cannabinoids predominate in the plasma of cattle fed non-fermented IH products. This suggests that they are readily absorbed from the rumen but are eliminated more slowly from the plasma than neutral cannabinoids, potentially due to ion trapping, protein binding, or lower lipophilicity. To this point, Irawan et al. (2025) calculated increased absorption rates of CBCA and CBDA

compared to their neutral forms and Wagner et al. (2022) reported a much lower milk:plasma ratio for THCA compared to 9-THC or the other neutral cannabinoids detected. Similarly, in dogs, CBDA and THCA appear to be more readily absorbed than CBD and 9-THC, respectively (Wakshlag et al., 2020).

Like the cannabinoid profiles we report, the cannabinoid elimination data from this study is consistent with prior literature. Fruge et al. (2025) found that CBD, CBDA, and THCA rapidly depleted to very low plasma concentrations within 96 h of the last feeding of IH leaves. In a study evaluating hempseed cake, Smith et al. (2023) also reported undetectable plasma CBDA/THCA and CBDVA by day 2 of withdrawal. After a single dose of IH flowers, Kleinhenz et al. (2020a) observed rapid depletion of CBDA within 96 h, calculating a mean residence time of 30.6 h. In that study, CBCA and THCA were undetectable after 72 h and CBDVA concentrations were approximately 1 ng/mL. In the subsequent 14-day feeding study, the authors reported all cannabinoids except CBDA were undetectable after day 2 of withdrawal; CBDA was undetectable on day 5 (Kleinhenz et al., 2022).

In the present study, most of the acidic and neutral cannabinoids were eliminated from the plasma by 7 days after final IH administration (the first blood collection after the last dose). However, two metabolites, CBD-7-acid and THC-acid-glu, were detected 7 and 14 days after the final dose. The FDA has a published interest in the inactive metabolite CBD-7-acid, as it reaches concentrations considerably higher than the parent, CBD, and has a much longer elimination half-life of up to 33 h (FDA CDER, 2017, Taylor et al., 2018, FDA CDER, 2023). In horses, CBD-7-acid elimination is also prolonged, with one study reporting a terminal elimination half-life of 79.85 h (Eichler et al., 2023). In humans, THC-acid-glu is the primary 9-THC metabolite excreted in the urine and has a much slower elimination than the parent compound, being

detected up to 19 days in frequent marijuana users (Ellis et al., 1985, Grotenhermen, 2003, Irawan et al., 2025). It also has been detected in plasma at similar or higher concentrations than 9-THC, particularly after oral marijuana exposure, and has a slower elimination rate than its precursor compounds (9-THC, THC-11-OH, and THC-acid) (Newmeyer et al., 2016).

Considering that both CBD-7-acid and THC-acid-glu have extended elimination phases in humans and other species, it is not surprising that we detected them longer than other cannabinoids. However, it is unexpected that THC-acid-glu was not detected prior to day 70 in any group but was detected in all groups—even those not receiving IH—on days 70 and 77, with the highest mean concentration observed in the CTC group. The maximum individual concentration of THC-acid-glu observed was 10.8 ng/mL (COMBO group). As mentioned above, in humans, THC-acid-glu is usually detected at higher concentrations in plasma than 9-THC. In addition, we would expect there to be detectable concentrations of THC-acid, the immediate precursor of THC-acid-glu. However, THC-acid was not above the limit of detection in any sample. Similarly, 9-THC was not detected above the LLOQ in any sample from the HEMP or COMBO groups, but one animal in the CTC group had detectable concentrations near the LLOQ (1.3 ng/mL detected vs. 1 ng/mL LLOQ). That same sample had THCA detected at a concentration similar to the HEMP and COMBO groups. The unexpected and sporadic nature of the THC-acid-glu and 9-THC findings begs the question whether the positive samples we identified were true positives or if they potentially represent contamination, interference, or noise from the matrix or assay. Given that all THC-acid-glu positives were from the same timepoints across all groups and were relatively close to the LLOQ, the authors believe compound interference or contamination (of the mass spectrometer or samples) is most likely responsible for the THC-acid-glu findings, as opposed to being a true positive. Considering that THC-acid-

glu elutes early from the column, matrix interference is likely playing a major role in the positive samples. Similar explanations are feasible for the positive 9-THC and THCA finding in the single sample an animal in the CTC group. However, it is also possible that there was exposure to those cannabinoids in the feces or urine of HEMP or COMBO animals. Transference of residues in urine or feces to unexposed animals has been reported for non-steroidal anti-inflammatory drugs in pigs and horses (Popot et al., 2007, Hairgrove et al., 2019, Bates et al., 2020). Similar to human literature on disposition of cannabinoids (Grotenhermen, 2003), previous literature in cattle receiving IH has reported high levels of fecal and urine excretion (Addo et al., 2023, Smith et al., 2023, Fruge et al., 2025, Irawan et al., 2025). Specifically, Fruge reported large standard deviations in their fecal cannabinoid measurements, indicating differences in individual animal's cannabinoid metabolism. The steer with detectable 9-THC and THCA may have been exposed to feces or urine from an animal with a particularly high fecal cannabinoid excretion rate. The authors believe this situation is less likely than the early elution or sample contamination scenarios, since there were no other samples with 9-THC concentrations above the LLOQ and no other CTC or PLBO samples with detectable THCA.

While the THC-acid-glu and 9-THC findings may be false positives, we believe the CBD-7-acid findings are reliable due to the precedent set by previous literature and the consistently positive samples throughout the feeding period. The authors believe that environmental contamination and residue transfer scenario is the most plausible reason for consistent detection of CBD-7-acid and CBDA in the PLBO and CTC animals. In addition, it appears that concurrent administration of CTC may increase CBD-7-acid concentrations, potentially due to changes in cytochrome P450 (CYP) isoform expression or activity. The main CYP isoenzymes critical to CBD metabolism in humans are CYP3A4 and CYP2C19 (Jiang et

al., 2011, Gaston and Friedman, 2017). While the role of CYP in CBD metabolism in bovines has not been established, four bovine CYP3A isoenzymes have been previously described, some of which correlate to human CYP3A4 (Zancanella et al., 2010). Furthermore, tetracycline has been shown to inhibit human CYP3A4 *in vitro* (Zhao and Ishizaki, 1997) and a study in pigs (Hu et al., 2016) reported variable risk of CYP2 and CYP3 inhibition by oxytetracycline, chlortetracycline, and doxycycline. Future work should confirm our findings and investigate potential risks of coadministration of veterinary drugs and IH in cattle.

Although we detected CBD-7-acid up to 14 d after final administration, we cannot accurately estimate half-life from our results. Determining the half-life and other pharmacokinetic parameters would enable the application of CBD-7-acid for exposure time estimation. Development of a rapid analytical method for this cannabinoid could provide a useful tool for quickly identifying exposed cattle for further testing. However, caution is warranted if an analytical method is developed for this purpose. The present study consistently identified CBD-7-acid in cattle not receiving IH, possibly secondary to exposure to urine and feces from cattle in the HEMP or COMBO groups. Thus, any future surveillance strategy should employ either a second, confirmatory test or should incorporate cut-off values that would exclude animals with detectable concentrations due to environmental contamination from exposed animals or should recommend confirmatory testing prior to any regulatory actions.

Limitations of this study include the small sample size and lack of follow-up past 14 days after final IH administration. There are no IH products approved in cattle and thus there is no tolerance for any cannabinoid in cattle tissues or plasma. If further regulatory discussions deem CBD-7-acid or THC-acid-glu of importance for surveillance measures, additional studies should be performed with a longer follow-up period. Additionally, a relatively low dosage of

cannabinoids was used in this study compared to previous feeding trials (Wagner et al., 2022, Fruge et al., 2025, Irawan et al., 2025). Longer durations and higher inclusion levels than what was used in the present study may result in even more prolonged depletion of cannabinoids in plasma. The authors also recognize the severe limitations of the treatment allocation for the original study design, as experimental vaccine status was a confounder with IH treatment. This restricted assignment scheme was chosen out of a desire to minimize unnecessary animal waste, considering that this was pilot data to be used to generate further hypotheses. For the purposes of this report, the confounder of vaccine status was not relevant, as we only compared the HEMP and COMBO groups, which both received the vaccine, and were merely reporting descriptive statistics for the CTC and PLBO groups.

In the present study, we observed that acidic cannabinoids represented a majority of plasma cannabinoids. We detected two cannabinoid metabolites, CBD-7-acid and THC-acid-glu, at 14 days after final IH administration and believe that CBD-7-acid could be a reliable indicator of prior IH exposure for use in future surveillance measures. These results will inform decisions regarding surveillance and interpretation of samples collected from cattle tested for cannabinoids or IH exposure.

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Agriculture. Any opinions, findings, conclusions, or recommendations expressed in this

publication are those of the author(s) and should not be construed to represent any official

USDA, FDA, or U.S. Government determination or policy.

Figure 5.1. Concentrations of CBD-7-acid, CBDA, CBDVA, and THCA (back transformed logarithmic mean ± SE) in Holstein steers administered IH flowers (HEMP; 5.5 mg/kg/d CBDA) or IH flowers and CTC (COMBO, CTC at 1.1 mg/kg/d) by mouth once daily for 63 days.[12]

Plasma cannabinoids

Table 5.1. Plasma cannabinoid concentrations (median; 95% CI) in steers administered IH inflorescence with or without CTC.

	Cannabinoid	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	LLOQ, ng/mL
HEMP	CBCA	2.6 (0, 6.7)	0 (0, 6.2)	5.4 (1.2, 8.2)	3 (0, 6.6)	3.4 (2.5, 4.6)	2.6 (0, 6.2)	2.5 (0, 5.4)	4.4 (2.0, 7.8)	0 (0, 4.4)	N/D	N/D	2.5
	CBD-7-acid	287 (156, 416)	370 (129, 646)	523 (121, 944)	297 (110, 536)	304 (136, 481)	630 (309, 961)	477 (321, 594)	657 (76, 1,125)	866 (0, 1,866)	601 (0, 1,358)	41 (0, 110)	1
	CBD	1 (0, 2.3)	0 (0, 1.9)	0	N/D	N/D	N/D	N/D	1.4 (0.3, 2.6)	N/D	N/D	N/D	1
	CBDA	92 (0, 290)	131 (0, 309)	154 (36, 304)	53 (33, 75)	123 (32, 197)	83 (29, 138)	70 (41, 98)	113 (0, 356)	52 (1.1, 117)	N/D	N/D	2.5
	CBDVA	2.7 (0, 8.0)	3.6 (0, 9.5)	3.2 (0, 7.8)	2.8 (0, 6.2)	4.9 (0.6, 8.8)	3.5 (0.6, 7.7)	0 (0, 7.2)	4.1 (0, 9.0)	4.3 (1.2, 7.4)	N/D	N/D	2.5
	CBGA	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0 (0, 4.4)	N/D	N/D	N/D	2.5
	CBLA	0 (0, 4.9)	N/D	3 (0, 7.6)	N/D	0 (0, 4.8)	N/D	N/D	3.4 (2.2, 5.2)	N/D	N/D	N/D	2.5
	THCA	6.5 (1.4, 12)	8 (2.2, 13)	6.8 (5.2, 8.8)	4.5 (3.5, 5.8)	7.9 (5.2, 9.6)	5.5 (1.4, 9.9)	4.4 (4.2, 4.6)	7.1 (2.5, 15)	5 (1.8, 9.3)	N/D	N/D	1
	THCV	N/D	0 (0, 1.9)	1.4 (1.3, 1.6)	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	1
	THCVA	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0 (0, 4.6)	0 (0, 4.6)	N/D	N/D	2.5
	THC-acid-glu	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	2.1 (0, 10.9)	1
COMBO	CBCA	3 (1.4, 5.5)	4.5 (0.5, 8.0)	3.8 (1.7, 5.9)	2.6 (0, 6.0)	4.3 (2.2, 6.2)	2.9 (1.7, 4.8)	3.1 (1.9, 4.3)	5.1 (2.3, 8.1)	0 (0, 4.6)	N/D	N/D	2.5
	CBD-7-acid	392 (240, 588)	558 (228, 863)	770 (199, 1,224)	435 (148, 650)	466 (0, 1,606)	661 (0, 1,534)	683 (140, 1,047)	902 (180, 1,610)	995 (21, 2,246)	542 (0, 1,702)	157 (0, 296)	1
	CBD	1.1 (0.8, 1.5)	0 (0, 2.7)	N/D	N/D	N/D	0	1.1 (0.6, 1.7)	1.4 (0.7, 2.3)	1.1 (0, 2.5)	N/D	N/D	1
	CBDA	140 (8.9, 237)	141 (87, 199)	146 (0, 282)	43 (20, 68)	78 (45, 100)	92 (49, 122)	80 (31, 137)	155 (0, 393)	44 (0, 156)	N/D	N/D	2.5
	CBDVA	3.3 (0, 9.2)	3.8 (1.0, 6.3)	3.1 (0, 9.7)	0 (0, 7.8)	4.9 (1.7, 7.2)	4.8 (0.6, 7.9)	3.2 (0, 8.2)	3.9 (0.8, 8.8)	6.4 (0.5, 10)	N/D	N/D	2.5
	CBLA	0 (0, 5.8)	3.1 (0, 6.6)	2.7 (0, 5.9)	N/D	2.6 (0, 6.0)	N/D	0 (0, 4.8)	3.8 (0, 9.2)	N/D	N/D	N/D	2.5
	THCA	5.7 (2.5, 11)	9.2 (5.4, 12)	5.6 (1.5, 12)	4.5 (1.1, 8.0)	6.3 (4.5, 9.0)	4.3 (1.7, 7.3)	5.1 (1.1, 9.1)	9.6 (1.7, 17)	4.8 (2.8, 6.4)	N/D	N/D	1
	THCV	N/D	1.1 (0, 3.5)	1.6 (0, 3.7)	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	1
	THCVA	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0 (0, 5.3)	N/D	N/D	2.5
	THC-acid-glu	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	0 (0, 2.7)	2.4 (0, 17.6)	1

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