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Andressa Nunes Mouta

Perfil farmacocinético dos metabólitos ativos da dipirona, 4-metilaminoantipirina (MAA) e 4-aminoantipirina (AA), isolados e associados ao tramadol em cães

MOSSORÓ

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Tese apresentada ao Doutorado em Ciência Animal do Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi Árido (UFERSA), como requisito para obtenção do título de Doutora em Ciência Animal.

Linha de Pesquisa: Sanidade Animal

Orientadora: Valéria Veras de Paula, Profa. Dra.

MOSSORÓ

2026

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Existe um dito popular, uma paráfrase de um poema de Robert Frost: A única saída é atravessar. A única maneira de se chegar ao outro lado do túnel é passando por dentro dele, e não o rodeando.

(Lori Gottlieb)

PERFIL FARMACOCINÉTICO DOS METABÓLITOS ATIVOS DA DAPIRONA, 4-METILAMINOANTIPIRINA (MAA) E 4-AMINOANTIPIRINA (AA), ISOLADOS E ASSOCIADOS AO TRAMADOL EM CÃES

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RESUMO: Esta pesquisa investigou o perfil farmacocinético dos metabólitos ativos da dipirona, 4-metilaminoantipirina (MAA) e 4-aminoantipirina (AA), em cães, avaliando tanto a administração isolada quanto a sua associação com o tramadol. Foram realizados dois estudos experimentais utilizando cães sem raça definida. Na primeira etapa, 11 cães SRD receberam dipirona intravenosa ($25 \text{ mg} \cdot \text{kg}^{-1}$). Na segunda etapa, 9 cães foram utilizados em delineamento crossover para comparar a dipirona isolada ($25 \text{ mg} \cdot \text{kg}^{-1}$) em associação ao tramadol ($2 \text{ mg} \cdot \text{kg}^{-1}$), por via intravenosa. Em ambos, coletou-se amostras para obtenção de plasma sanguíneo nos tempos: 0 (basal), 5, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36 e 48 horas. As análises foram feitas por cromatografia líquida de ultra eficiência acoplada a espectrometria de massas (UPLC-MS/MS) e os parâmetros farmacocinéticos calculados via PKSolver 2.0. A análise estatística incluiu Análise de Componentes Principais (ACP) para a primeira etapa, e testes de t e Mann-Whitney na etapa 2 ($p < 0,05$), correlacionados via Python®. No estudo 1, foram identificados dois fenótipos metabólicos para MAA: metabolizadores lentos (ML) e normais/rápidos (MN). O grupo ML apresentou $T_{1/2}$ de $44,44 \pm 11,74\text{h}$ e MRT de $32,62 \pm 16,53\text{h}$, enquanto o grupo MN apresentou $11,25 \pm 5,37\text{h}$ e $7,44 \pm 4,25\text{h}$, respectivamente. No estudo 2, a interação farmacocinética revelou que o tramadol alterou significativamente o $C_{\text{máx}}$ e C_0 do MAA. Para a AA, houve aumento na biodisponibilidade (diferenças significativas em ASC_{0-t} , $ASC_{0 \rightarrow \infty}$, Cl e $MRT_{0 \rightarrow \infty}$). Observou-se ainda que a dipirona interferiu na conversão do tramadol em seu metabólito ativo M1. Os metabólitos da dipirona permaneceram detectáveis por 48h, sugerindo concentrações eficazes para inibição de cicloxigenase. Concluiu-se que a dipirona apresenta variabilidade metabólica fenotípica em cães. Sua associação com tramadol aumenta a exposição à AA e interfere na ativação do tramadol em M1, indicando que a eficácia analgésica do protocolo depende de interações metabólicas complexas, sem potencializar efeitos adversos relacionados ao MAA.

Palavras-Chave: cromatografia; fenótipo metabólico; interação farmacocinética; metabólitos.

PERFIL FARMACOCINÉTICO DOS METABÓLITOS ATIVOS DA DAPIRONA, 4-METILAMINOANTIPIRINA (MAA) E 4-AMINOANTIPIRINA (AA), ISOLADOS E ASSOCIADOS AO TRAMADOL EM CÃES

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ABSTRACT: This research investigated the pharmacokinetic profile of the active dipyrone metabolites, 4-methylaminoantipyrine (MAA) and 4-aminoantipyrine (AA), in dogs, evaluating both isolated administration and interaction with tramadol. Two experimental studies were conducted using mixed-breed dogs. In the first stage 11 mixed-breed dogs received intravenous dipyrone ($25 \text{ mg} \cdot \text{kg}^{-1}$). In the second stage, 9 dogs were used in a crossover design to compare isolated dipyrone ($25 \text{ mg} \cdot \text{kg}^{-1}$) in association with tramadol ($2 \text{ mg} \cdot \text{kg}^{-1}$), intravenous. In both studies, blood plasma samples were collected at 0 (baseline), 5, 15, 30 and 45 min, as well as at 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36 and 48 hours. Analyses were performed using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) and pharmacokinetic parameters were calculated via PKSolver 2.0. Statistical analysis included Principal Component Analysis (PCA) for Study 1, and t-tests and Mann-Whitney tests for Study 2 ($p < 0.05$), with correlations performed via Python®. In Study 1, were identified two metabolic phenotypes for MAA: slow metabolizers (SM) and normal/rapid metabolizers (NM). The SM group presented $T_{1/2}$ of $44.44 \pm 11.74 \text{ h}$ and MRT of $32.62 \pm 16.53 \text{ h}$, while the NM group presented $11.25 \pm 5.37 \text{ h}$ and $7.44 \pm 4.25 \text{ h}$, respectively. In Study 2, the pharmacokinetic interaction revealed that tramadol significantly altered the C_{max} and C_0 of MAA. For AA, there was an increase in bioavailability (significant differences in AUC_{0-t} , $AUC_{0 \rightarrow \infty}$, Cl e $MRT_{0 \rightarrow \infty}$). It should also be noted that dipyrone interfered with the conversion of tramadol to its active metabolite M1. Dipyrone metabolites were detectable for 48 hours, providing injectable vitamins for cyclooxygenase inhibition. It is concluded that dipyrone exhibits phenotypic metabolic variability in dogs. Its association with tramadol increases AA exposure and interferes with tramadol activation at M1, suggesting that the analgesic efficacy of the protocol depends on complex metabolic interactions, without potentiating adverse effects related to MAA.

Keywords: chromatography; pharmacokinetic interaction; metabolites; metabolic phenotype.

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CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

O controle da dor em pequenos animais é de fundamental importância na clínica (GRUEN *et al.*, 2022). O uso de associações farmacológicas com analgésicos opioides, não opioides e anti-inflamatórios não esteroidais (AINE) representa a prática mais rotineiramente empregada para tratamento de afecções que culminam em dor aguda em cães e gatos (BRADBROOK; CLARK, 2018; IMAGAWA *et al.*, 2011). Apesar de usadas, muitas dessas associações são adotadas com informações limitadas acerca de possíveis interações farmacocinéticas.

A dipirona é um analgésico que apresenta efeitos espasmolíticos e antipiréticos, indicado para tratamento da dor aguda em humanos e animais (BAUMGARTNER *et al.*, 2009). Embora previamente classificada como AINE, devido à sua ação de inibir a isoenzima ciclooxigenase (COX), de forma não seletiva, este fármaco apresenta fraca ação anti-inflamatória (CHANDRASEKHARAN *et al.*, 2002). Seu uso é proibido em países como os Estados Unidos, Japão e Austrália, devido relatos de efeitos colaterais relacionados à agranulocitose, no entanto, alguns países da Europa, Ásia e América do Sul, permanecem comercializando este fármaco (HEDENMALM; SPIGSET, 2002; JASIECKA; MAŚLANKA; JAROSZEWSKI, 2014). Essa proibição de uso em muitos países desenvolvidos impactou diretamente no menor número de pesquisas envolvendo a farmacocinética da dipirona em animais.

O tramadol é um analgésico opioide amplamente utilizado para tratamento da dor aguda em humanos e animais, com ação central. Este agente é classificado como um opioide atípico, por promover analgesia decorrente não apenas da atuação em receptores opioide tipo μ (MOP), como também promove uma ação monoaminérgica de inibição da recaptação de noradrenalina e serotonina (GROND; SABLITZKI, 2004; BRAVO; MICO; BERROCOSO, 2017).

A farmacocinética auxilia na escolha do regime de doses e avaliação geral de como o organismo afeta o comportamento de um fármaco (CURRIE, 2018). A metabolização é a fase a qual o fármaco passa por reações enzimáticas e modificações, resultando na formação de metabólitos inativos ou, no caso de pró-fármacos, na conversão para suas formas ativas (URSO; BLARDI; GIORGI, 2002). As enzimas do sistema citocromo P450 (CYP) exercem papel central no metabolismo e na eliminação de inúmeros fármacos utilizados na prática clínica veterinária, sendo reconhecidas como um dos principais determinantes da variabilidade farmacocinética entre indivíduos (COURT, 2013).

Tanto a dipirona como o tramadol dependem do metabolismo via CYP para sua metabolização completa. A dipirona sofre hidrólise química rápida para 4-metilaminoantipirina (MAA), a qual é posteriormente metabolizada no fígado pelas enzimas de CYP em 4-aminoantipirina (AA), sendo ambos ativos (LUTZ, 2019). Enquanto o tramadol é biotransformado no fígado em diversos metabólitos, com destaque para o O-desmetiltramadol (M1), sendo seu metabólito ativo (e principal metabólito descrito na literatura por ser responsável pela analgesia nas diferentes espécies), além dos metabólitos o N-desmetiltramadol (M2) e N,O-didesmetiltramadol (M5) (MIOTTO *et al.*, 2017).

No que se refere aos estudos farmacocinéticos em cães, o tramadol tem sido amplamente investigado, principalmente em razão das controvérsias quanto ao poder analgésico deste fármaco e a formação do metabólito ativo M1 nessa espécie (KUKANICH; PAPICH, 2004; MCMILLAN *et al.*, 2008; GIORGI *et al.*, 2010; KUKANICH *et al.*, 2011). Em contraste, há apenas um estudo relatando o perfil farmacocinético da dipirona em cães (GIORGI *et al.*, 2018) e um avaliando as concentrações plasmáticas de MAA e AA (KALCHOFFNER *et al.*, 2015), apesar do seu uso clínico frequente, tanto de forma isolada quanto em associação com outros analgésicos (IMAGAWA *et al.*, 2011; ZANUZZO *et al.*,

2015; MANSFIELD; BETHS, 2015; DONATI *et al.*, 2021; FLÔR *et al.*, 2013; TEIXEIRA *et al.*, 2013).

Diante desse contexto, não existem estudos que avaliem como a associação de dipirona e tramadol interfere na farmacocinética dos metabólitos ativos da dipirona em cães, embora existam em outras espécies como ratos (Moreno Rocha *et al.*, 2016) e jumentos (Araújo-Silva *et al.*, 2024), o que representa uma lacuna relevante, considerando o potencial impacto clínico dessa interação sobre a eficácia analgésica.

Diante da importância desses agentes na clínica de pequenos animais e da escassa literatura científica acerca do perfil farmacocinético detalhado da dipirona e de sua associação ao tramadol em cães, o objetivo desta pesquisa foi avaliar o perfil farmacocinético da dipirona isolada e associada ao tramadol em cães hípidos. Essa investigação visa preencher lacunas críticas sobre o comportamento biológico desses fármacos na espécie, contribuindo para o entendimento das possíveis interações farmacocinéticas, fornecendo informações científicas relevantes para o uso clínico mais seguro e racional da associação na rotina da clínica de pequenos animais.

A hipótese da pesquisa foi que a administração da dipirona associada ao tramadol em cães é capaz de modificar o perfil farmacocinético dos metabólitos ativos da dipirona, quando comparada à sua administração isolada, devido à potencial competição enzimática pelo sistema citocromo P450. Ademais, pode existir variabilidade de metabolização entre cães de um mesmo grupo, podendo ocorrer diferentes perfis de metabolização (rápidos ou lentos), o que influenciaria diretamente a exposição sistêmica aos metabólitos dos fármacos. Além disso, a associação poderia atingir concentrações plasmáticas suficientes para promoção da analgesia em cães, possivelmente com um maior tempo de meia vida dos fármacos.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar e comparar o perfil farmacocinético dos metabólitos ativos da dipirona 4-metilaminoantipirina (MAA) e 4-aminoantipirina (AA), após administração única por via intravenosa, de forma isolada e em associação ao tramadol, em cães hígidios.

2.2 OBJETIVOS ESPECÍFICOS

Determinar e comparar os parâmetros farmacocinéticos dos metabólitos ativos da dipirona, 4-metilaminoantipirina (MAA) e o 4-aminoantipirina (AA) na dose 25mg.kg^{-1} , por via intravenosa em cães.

Determinar os parâmetros farmacocinéticos do tramadol e dos seus metabólitos O-desmetiltramadol (M1), N-desmetiltramadol (M2) e N,O-didesmetiltramadol (M5) na dose de 2 mg.kg^{-1} associado à dipirona na dose de 25 mg.kg^{-1} , por via intravenosa em cães.

Validar metodologia analítica e bioanalítica por cromatografia líquida de ultraeficiência acoplada a espectrometria de massas (UPLC-MS/MS) para quantificação da dipirona e seus metabólitos, 4-Metilaminoantipirina (MAA) e o 4-aminoantipirina (AA), em plasma sanguíneo canino.

Validar metodologia analítica e bioanalítica por cromatografia líquida de ultraeficiência acoplada a espectrometria de massas (UPLC-MS/MS) para quantificação de tramadol e seus metabólitos, O-desmetiltramadol (M1), N-desmetiltramadol (M2) e N,O-didesmetiltramadol (M5) em plasma sanguíneo canino.

Monitorar a ocorrência de efeitos adversos e possíveis alterações comportamentais decorrentes da administração da dipirona isolada e em associação ao tramadol.

3. HIPÓTESES

- Interação Farmacocinética: A associação de dipirona e tramadol altera o perfil farmacocinético dos metabólitos ativos da dipirona (MAA e AA) por meio de competição enzimática pelo sistema citocromo P450.
- Variabilidade Fenotípica: Existe variabilidade metabólica individual em cães sem raça definida (SRD), permitindo a classificação dos animais em diferentes fenótipos de metabolização.
- Concentrações plasmáticas: A coadministração dos fármacos resulta em concentrações plasmáticas compatíveis com a eficácia analgésica, promovendo um incremento no tempo de meia-vida dos compostos.

4. REFERENCIAL TEÓRICO

4.1 DOR E ANALGESIA EM CÃES

O conceito de dor foi definido em 1979, pela Associação Internacional para o Estudo da Dor (IASP, 1979) e recentemente foi revisado, em 2020, sendo considerada: “uma experiência sensitiva e emocional desagradável, associada, ou semelhante àquela associada, a uma lesão tecidual real ou potencial”. A classificação da dor é bastante ampla, podendo ser relacionada à sua origem, a qual pode ser subdividida em neuropática ou nociceptiva, a qual a dor neuropática ocorre quando há uma lesão no sistema nervoso somatossensorial, incluindo neuropatias periféricas ou lesão medular, enquanto a dor nociceptiva está relacionada à lesão somática ou visceral (ATTAL, 2019). Com relação à sua duração, pode ser considerada aguda, quando tem limitada duração e está relacionada à lesão repentina, ou crônica, quando apresenta uma duração maior do que a esperada (BELL, 2018).

O processo fisiológico da dor, compreende etapas consecutivas e integradas, chamadas de transdução, transmissão, modulação e percepção. Durante um processo doloroso, ocorre ativação de nociceptores (terminações nervosas livres de fibras mielínicas A- δ e amielínicas C), que são encontrados na pele, vasos sanguíneos e fibras do músculo esquelético,

os quais estes estímulos ambientais são transformados em sinais elétricos chamados de potenciais de ação, definindo-se então a transdução. Em seguida, esses impulsos elétricos são transmitidos para a medula espinhal através de nervos periféricos (transmissão). Nesta região, os impulsos sofrem influência de vias inibitórias ou facilitatórias (modulação) antes de serem projetados para os centros encefálicos superiores para o reconhecimento do estímulo (percepção) (FANTONI; MASTROCINQUE, 2010; HERNANDEZ-AVALOS *et al.*, 2019). A falha nos mecanismos de modulação pode resultar em sensibilização central, que é uma alteração no estado funcional dos neurônios e das vias nociceptivas, podendo ser causada pelo aumento da excitabilidade da membrana ou diminuição da inibição nesse sistema (WOOLF, 2011).

A dor pode promover efeitos deletérios graves aos animais, sendo de suma importância o seu adequado tratamento, de acordo com sua classificação, visando melhorias no bem-estar animal (BELSHAW, YEATES, 2018). O manejo da dor deve ser baseado na identificação precoce e na utilização de analgesia multimodal, que consiste na utilização de fármacos de classes diferentes para atuar em múltiplos sítios das vias nociceptivas. A escolha dos analgésicos deve considerar a espécie, a condição clínica e a segurança dos fármacos (GRUEN *et al.*, 2022).

Associações analgésicas utilizando agentes opioides e não opioides são muito utilizadas, visando o controle da dor (LORENA *et al.*, 2016). Os pacientes que experimentam dor necessitam da utilização de terapias com a associação de fármacos de diferentes grupos, visando a redução de seus efeitos maléficos, sendo a associação de analgésicos opioides, não opioides e AINES normalmente as mais escolhidas quando se objetiva o tratamento da dor em cães (PIEPER, 2016). Diante disso, conhecer a farmacocinética dos analgésicos é fundamental para o controle da dor, uma vez que a eficácia analgésica está ligada à manutenção de concentrações plasmáticas dentro da janela terapêutica, evitando-se tanto falha terapêutica como toxicidade (VOLLES; MCGORY, 1999).

Dentre os analgésicos não opioides utilizados na clínica de pequenos animais, destaca-se a dipirona, frequentemente associada a opioides como o tramadol. No entanto, embora essa associação seja rotineira, a literatura tem poucos dados sobre como a interação entre ambos afeta a farmacocinética de seus metabólitos.

4.2 DIPIRONA

A dipirona, também chamada de metamizol, é um fármaco que tem ação analgésica, antipirética e espasmolítica, sendo um composto derivado das pirazolonas, introduzido no mercado em 1922 (GARCIA DEL CAMPO *et al.*, 2021). Seu mecanismo de ação ainda não está completamente elucidado, sendo atribuído principalmente aos seus metabólitos ativos, como o 4-metilaminoantipirina (MAA), que promove inibição das enzimas COX-1 e COX-2, de forma menos intensa que os AINEs, e o 4-aminoantipirina (AA), que é capaz de reduzir comportamento de dor induzido pelas prostaglandinas (PGE2) (CASCORBI, 2021). Essa inibição enzimática de COX resulta na redução da síntese de prostaglandinas e prostaciclina, contribuindo para os efeitos analgésicos e antipiréticos. A dipirona também pode atuar por mecanismos adicionais, incluindo a modulação da COX-3 e a participação de vias envolvendo receptores de potencial transitório de anquirina (TRPA-1), o sistema canabinoide e o sistema opioide endógeno, embora a relevância clínica desses mecanismos ainda esteja em investigação (CHANDRASEKHARAN *et al.*, 2002; NASSINI *et al.*, 2015; LUTZ, 2019). Além da inibição enzimática, a dipirona também atua na excitabilidade dos neurônios sensoriais (CASCORBI, 2021).

Por muito tempo, a dipirona foi considerada como um anti-inflamatório não esteroide, no entanto, as classificações recentes a definem como analgésico não opioide. Seu uso é indicado para tratamento da dor aguda após lesões ou cirurgias, cólicas, tumores, além de tratamento da febre e boa eficácia para analgesia visceral (SCHÜTTER; TÜNSMEYER; KÄSTNER, 2016; CHATZIMANOUIL *et al.*, 2023). Na clínica de pequenos animais, sua utilização é valorizada em situações nas quais se busca analgesia eficaz com menor interferência nos mecanismos inflamatórios periféricos, quando comparada aos AINEs. Devido à possibilidade de ser administrada via intravenosa, ter baixo custo e relação risco/benefício favorável, seu uso é frequente em ambientes hospitalares (BRINKMAN *et al.*, 2025). O uso clínico frequente reforça a necessidade de compreensão aprofundada da farmacocinética.

A dipirona pode apresentar alguns efeitos adversos, principalmente em uso prolongado, como agranulocitose e anemia aplásica em humanos, bem como efeitos individuais de lesão hepática em diferentes indivíduos, embora seja um fármaco relativamente seguro (BASAK *et al.*, 2010; HOFFMANN; BANTEL; JOBSKI, 2020). Estes efeitos adversos graves e, principalmente, a não comercialização da droga são os responsáveis pela ausência da dipirona

em muitos países como Reino Unido, Canadá e Estados Unidos, embora em países como Espanha, México e alguns na América do Sul, incluindo o Brasil, o fármaco esteja disponível para uso (CHATZIMANOUIL *et al.*, 2023). Apesar do risco da agranulocitose em humanos, ainda não existem relatos desse acontecimento em medicina veterinária (KALCHOFNER *et al.*, 2015). Em uma revisão sistemática recente analisando a segurança da dipirona em humanos, os resultados da pesquisa demonstraram sua segurança, com menor incidência de efeitos adversos em comparação a AINEs como o paracetamol e ácido acetilsalicílico (ELEUTÉRIO *et al.*, 2024).

Com relação à sua biotransformação, sabe-se que ocorre um processo sequencial complexo. Inicialmente a dipirona sofre hidrólise química rápida para seu metabólito ativo principal, o 4-metilaminoantipirina (MAA) (BACHMANN *et al.*, 2022). O MAA é então metabolizado por N-desmetilação, mediado por CYP em 4-aminoantipirina (AA), que também é um metabólito ativo. O AA segue via C-oxidação, transformando-se em 4-formilaminoantipirina (FAA), metabólito inativo, ou passa por acetilação, via N-acetiltransferase (NAT), resultando em 4-acetilaminoantipirina (AAA), que também é inativo (LEVY *et al.*, 1995; BACHMANN *et al.*, 2022). Em um estudo recente foi relatado que a dipirona é um agente indutor moderado a forte das enzimas CYP3A4, CYP2B6 e CYP2C19 em humanos (BRINKMANN *et al.*, 2025).

Os estudos farmacocinéticos sobre esse medicamento em cães são escassos. Giorgi *et al.* (2018) avaliaram a farmacocinética de seus metabólitos, na dose de 25 mg·kg⁻¹ em diferentes vias de administração em cães, utilizando cromatografia líquida com detector ultravioleta (UV), de modo que o seu principal metabólito ativo, o MAA foi detectado em 5 minutos pela via intravenosa e em 30 minutos por via oral e permaneceu detectável por até 24 horas em ambas as vias, além da via intramuscular e retal. Além disso, a via intravenosa foi a que apresentou a concentração mais expressiva de MAA nos cães do estudo. Kalchofner *et al.* (2015) também avaliaram a concentração plasmática do MAA e AA de uma formulação oral de liberação lenta de dipirona em cadelas, 50 mg·kg⁻¹ no pós operatório de OH. Os metabólitos foram detectáveis por até 4 horas. Apesar da grande relevância desses estudos, os métodos analíticos empregados apresentam menor sensibilidade, o que pode limitar a detecção precisa dos metabólitos em concentrações mais baixas.

Embora os estudos de Giorgi *et al.* (2018) e Kalchofner *et al.* (2015) tenham estabelecido dados sobre as concentrações plasmáticas e o perfil farmacocinético da dipirona

em cães, limitações inerentes aos detectores utilizados no método do estudo podem restringir a compreensão de alguns pontos como a eliminação dos medicamentos e o tempo de quantificação dos metabólitos no organismo dos animais. A identificação precisa das concentrações plasmáticas, especialmente em níveis reduzidos, é um desafio que as tecnologias cromatográficas convencionais podem enfrentar ao analisar estes metabólitos em cães.

4.3 TRAMADOL

O tramadol é um analgésico opioide amplamente empregado no manejo da dor, atuando principalmente no sistema nervoso central (SUBEDI *et al.*, 2019; RUEL; STEAGALL, 2019). Trata-se de um opioide atípico, uma vez que seu efeito analgésico resulta tanto da ativação dos receptores opioides μ (MOP) quanto de sua ação monoaminérgica, caracterizada pela inibição da recaptação de norepinefrina e serotonina (GROND; SABLITZKI, 2004; BRAVO, MICO; BERROCOSO, 2017). Estudos que avaliam o uso do tramadol em cães para o tratamento de condições clínicas e no controle da dor pós e perioperatória estão amplamente descritos na literatura (MANSFIELD; BETHS, 2015; DONATI *et al.*, 2021).

Do ponto de vista farmacocinético, o tramadol sofre intensa biotransformação hepática por desmetilação, oxidação e conjugação hepática, formando pelo menos 30 metabólitos diferentes (BARBOSA *et al.*, 2016). Esse processo é mediado por enzimas CYP (principalmente CYP3A4, CYP2D6 e CYP2B6 em humanos e CYP2D15 em cães), originando primariamente o metabólito ativo O-desmetiltramadol (M1). Paralelamente, ocorre a N-desmetilação, formando o N-desmetiltramadol (M2), processo realizado por múltiplos CYPs, incluindo CYP2B11, CYP3A12 e CYP2C41. Ambos os metabólitos primários podem convergir para formação do N,O-didesmetiltramadol (M5), por metabolismo oxidativo adicional (BEAKLEY; KAYE; KAYE, 2015; VAZZANA *et al.*, 2015; MIOTTO *et al.*, 2017; DOMÍNGUEZ-OLIVA *et al.*, 2021). Esse metabolismo evidencia que a resposta analgésica ao tramadol depende fortemente da eficiência das vias metabólicas hepáticas, o que pode resultar em elevada variabilidade interindividual.

A literatura sobre o M1 ser o metabólito que se destaca por mediar os efeitos analgésicos, devido sua alta afinidade aos receptores opioides (200 a 300 vezes maior afinidade do que o tramadol) é muito descrita nos estudos em humanos (HENNIES *et al.*, 1988). Menciona-se que em cães, a formação do metabólito M1 é mais lenta do que em outras espécies, enquanto a produção do M2 é mais rápida, o que favorece o desvio metabólico para rotas não analgésicas (PEREZ JIMENEZ *et al.*, 2016a). Em estudos anteriores, as concentrações de M5 em cães foram encontradas cerca de 20 vezes acima das concentrações de M1 (GIORGI *et al.*, 2009; KUKANICH; PAPICH, 2011). Nos cães, todavia, ainda há lacunas sobre as informações acerca do M1. As variações na formação do metabólito podem ter relação com polimorfismos genéticos e inibição de CYP, principalmente a CYP2D15, que é a responsável pela formação do metabólito em cães (PEREZ JIMENEZ *et al.*, 2016b; DOMÍNGUEZ-OLIVA *et al.*, 2021).

Com relação aos efeitos adversos, foram relatados em cães e em outras espécies alterações como tremores musculares, sialorreia, euforia, sedação, distúrbios gastrointestinais e náusea, principalmente em doses mais altas (MOUTA *et al.* 2021; DOMÍNGUEZ-OLIVA *et al.*, 2021).

O tramadol é comumente administrado por via oral e intravenosa. As doses intravenosas variam entre 2 a 4 mg·kg⁻¹, sendo utilizado sozinho ou como parte de um protocolo multimodal (DOMÍNGUEZ-OLIVA *et al.*, 2021). As características farmacocinéticas do tramadol estão sendo amplamente estudadas nas diversas espécies animais, sendo os estudos em cães bastante diversos, demonstrando diferenças relacionadas à sua metabolização e à concentração do M1. McMillan *et al.* (2008) em seu estudo em cães utilizando diferentes doses pela via intravenosa (1, 2 e 4 mg·kg⁻¹), com cromatografia gasosa, constataram que apenas alguns animais do estudo conseguiram produzir o metabólito M1, e os que produziram, apresentaram quantidades próximas ao limite de quantificação mínima do método. O mesmo foi encontrado no estudo de Giorgi *et al.* (2009), utilizando a dose de 4 mg·kg⁻¹ por via intramuscular e intravenosa, com detector fluorescência, apresentou baixas concentrações de M1 nos animais.

Entretanto, em um estudo realizado por Kukanich *et al.* (2011), utilizando o tramadol por via oral em cães, todos os animais apresentaram a formação de M1, no entanto, também nas concentrações próximas ao limite mínimo de quantificação. Apesar de os estudos clínicos e farmacocinéticos existentes para tramadol em cães, ainda existem muitas contradições quanto à formação do metabólito M1. Os estudos farmacocinéticos permitem a

investigação dos perfis de disposição e eficácia farmacológica, entretanto, podem ser influenciados por condições relacionadas às características da metodologia do experimento ou clínicas (FAN; DE LANNOY, 2014).

As divergências observadas nos estudos farmacocinéticos disponíveis podem estar relacionadas a fatores individuais e metodológicos, como diferenças nas vias de administração, doses utilizadas e nos métodos analíticos adotados. A utilização de técnicas com menor sensibilidade pode comprometer a detecção de metabólitos ativos em baixas concentrações, influenciando a interpretação dos resultados farmacocinéticos. A aplicação da cromatografia líquida acoplada à espectrometria de massas (UPLC-MS/MS) tem se expandido significativamente na pesquisa clínica, sendo considerada essencial para o monitoramento terapêutico de fármacos e a identificação precisa de metabólitos (KIM; LEE; HUR, 2022).

4.4 SISTEMA CITOCROMO P450 (CYP) E VARIABILIDADE EM CÃES

As enzimas do CYP são responsáveis pela biotransformação de aproximadamente 50 a 60% dos medicamentos utilizados na clínica, incluindo analgésicos. Nesse contexto, os polimorfismos gênicos são os principais determinantes da variabilidade interindividual na resposta farmacológica. Na medicina veterinária, a farmacogenética, responsável por investigar a influência da composição genética na resposta aos fármacos, é uma área relativamente recente, porém vem apresentando avanços relevantes (JIMENEZ; ZHU; COURT, 2023).

Em cães, assim como em humanos, observa-se ampla variação interindividual na atividade das enzimas do CYP, o que pode resultar em respostas terapêuticas divergentes frente a uma mesma dose de um fármaco, variando desde eficácia reduzida, associada à elevada atividade enzimática e rápida depuração do fármaco, até efeitos excessivos ou tóxicos decorrentes de metabolismo lento e maiores concentrações plasmáticas (COURT, 2013). A resposta individual aos fármacos é resultado de uma complexa interação entre fatores fisiológicos, ambientais e genéticos, sendo o metabolismo hepático um dos principais determinantes da eficácia e segurança terapêutica. A variabilidade na atividade das enzimas de

CYP, observada entre espécies, raças e indivíduos, pode levar a diferenças marcantes nos perfis farmacocinéticos e farmacodinâmicos, com implicações clínicas relevantes (KARAKUS *et al.*, 2021).

Embora cães e humanos compartilhem a predominância das enzimas da subfamília CYP3A no fígado e nos intestinos, cães apresentam maior expressão relativa das isoformas CYP2D15 e CYP2B11 em comparação às isoformas humanas correspondentes CYP2D6 e CYP2B6, respectivamente (COURT, 2013). Considerando que, em humanos, a dipirona é metabolizada principalmente pelas isoformas CYP3A4, CYP2B6 e CYP2C19 (LUTZ, 2019), enquanto o tramadol depende majoritariamente das CYP3A4, CYP2B6 e CYP2D6 (MIOTO *et al.*, 2017) essa maior expressão das isoformas caninas pode contribuir para diferenças interespecíficas na biotransformação desses fármacos.

Polimorfismos do CYP2D6 são amplamente descritos na literatura humana e permitem classificar os indivíduos em diferentes fenótipos metabólicos (ultrarrápidos, intermediários ou lentos), os quais determinam variações relevantes na biotransformação de fármacos. Esses fenótipos influenciam diretamente a farmacocinética e a resposta clínica de medicamentos que dependem de ativação metabólica, como o tramadol, cuja conversão ao metabólito ativo (M1) é mediada principalmente pelo CYP2D6 em humanos. Como mencionado, em cães, o CYP2D15 é considerado o ortólogo funcional do CYP2D6 humano, e sua participação no metabolismo do tramadol já foi demonstrada, sugerindo que variações dessa enzima podem contribuir para diferenças interindividuais na formação do metabólito ativo (JIMENEZ; ZHU; COURT, 2023).

Esse fato reforça a necessidade de cautela na extrapolação de dados farmacocinéticos entre espécies e a importância de abordagens específicas para a medicina veterinária no uso racional de medicamentos. Estudos demonstram que mutações específicas em CYPs podem resultar em fenótipos metabólicos diferentes entre raças de cães e essas alterações enzimáticas podem impactar na eficácia terapêutica medicamentosa (DOMÍNGUEZ-OLIVA *et al.*, 2021).

4.5 ASSOCIAÇÃO DOS FÁRMACOS

A terapia farmacológica multimodal associando tramadol e dipirona foi descrita na literatura em diferentes espécies para tratamento de dores oncológicas, cirurgias como a ovariectomia (OH) e artrite (MORENO-ROCHA *et al.*, 2012; FLÔR *et al.*, 2013; TEIXEIRA *et al.*, 2013; MORENO-ROCHA *et al.*, 2016). No estudo de Teixeira *et al.* (2013), ao investigarem o uso do tramadol ($3 \text{ mg} \cdot \text{kg}^{-1}$) sozinho ou em combinação à dipirona ($30 \text{ mg} \cdot \text{kg}^{-1}$) ou meloxicam para manejo da dor pós-operatória de mastectomia unilateral, observou-se que o tramadol apresentou melhor resposta analgésica quando foi associado ao meloxicam ou a dipirona. No estudo de Flôr *et al.* (2013), os autores obtiveram como resposta que a associação de dipirona ($25 \text{ mg} \cdot \text{kg}^{-1}$) e tramadol ($2 \text{ mg} \cdot \text{kg}^{-1}$), combinada ou não com um AINE foi eficaz para tratamento de cães com dor oncológica crônica moderada a intensa. Espera-se com a associação dos fármacos que ocorram vantagens da utilização combinada, promovendo redução dos efeitos adversos de ambos, bem como aumento de efeitos antinociceptivos, devido ao sinergismo entre eles (LÓPEZ-MUÑOZ *et al.*, 2013).

A interação entre a dipirona e tramadol pode ser atribuída tanto às enzimas do CYP quanto a mecanismos farmacodinâmicos no sistema opioide. Moreno-Rocha *et al.* (2016) descreveram que a associação entre dipirona e tramadol em ratos resultou em efeito antinociceptivo superior ao uso isolado da dipirona, sugerindo interação farmacológica entre os fármacos, possivelmente relacionada ao sistema opioide. Os autores destacaram que, embora o mecanismo exato dessa interação não esteja completamente elucidado, a participação de vias comuns pode influenciar a resposta analgésica observada.

Araújo-Silva *et al.* (2024) avaliaram o perfil farmacocinético de duas doses diferentes de dipirona (10 e $25 \text{ mg} \cdot \text{kg}^{-1}$) associada a $2 \text{ mg} \cdot \text{kg}^{-1}$ de tramadol em jumentos. Os resultados indicaram que a dipirona aumentou a biodisponibilidade tanto do tramadol quanto do M1 de forma dose-dependente, de modo que a coadministração pode alterar a frequência da administração e a eficácia analgésica do tramadol nesta espécie.

Brinkmann *et al.* (2025) ao escreverem uma revisão de literatura recente sobre dipirona em humanos, reforçaram que as interações farmacocinéticas e farmacodinâmicas podem ter várias implicações na prática clínica. Como as enzimas CYP3A4 e CYP2B6 estão

relacionadas a cerca de 30% do metabolismo de mais da metade dos fármacos disponíveis, e pelo fato de a dipirona ser indutor das enzimas mencionadas, algumas associações podem ter consequências importantes. Os autores não mencionam o tramadol, entretanto, listam que alguns outros opioides como a metadona e o fentanil devem ser administrados com cautela quando associados à dipirona, sugerindo-se que se reduza a dose da dipirona ou fazer a titulação da dose do agente coadministrado.

Diante do exposto, observa-se que o uso clínico da combinação dipirona e tramadol em cães necessita de um suporte farmacocinético que avalie a influência mútua na biotransformação de ambos os fármacos. As evidências atuais limitam-se à eficácia analgésica da associação, sem considerar possíveis interferências na biotransformação. Assim, torna-se valiosa a aplicação da cromatografia líquida de ultra eficiência acoplada à espectrometria de massas (UPLC-MS/MS). Ademais, a investigação dos metabólitos M2 e M5 é fundamental, pois são poucos os estudos que quantificam estes metabólitos na espécie. É relevante entender o impacto da formação desses compostos nos perfis farmacocinéticos, uma vez que o M5 é um produto da biotransformação sequencial do M1 e do M2. Assim, monitorando as rotas pode-se identificar possíveis desvios, permitindo elucidar com precisão como a interação medicamentosa influencia a disponibilidade do fármaco e seus efeitos clínicos.

4.6. MÉTODOS ANALÍTICOS E VALIDAÇÃO FARMACOCINÉTICA

A cromatografia líquida de ultra eficiência (UPLC), é um método analítico fundamental na separação de misturas químicas complexas, operando por meio de bombas de alta pressão e colunas especializadas que garantem uma separação ideal dos analitos. Essa inovação tecnológica proporciona uma resolução cromatográfica significativamente aprimorada e tempos de análise mais rápidos em comparação aos sistemas convencionais. Além disso, a integração da UPLC com a espectrometria de massas permite a identificação e quantificação precisa de compostos, sendo amplamente aplicada no desenvolvimento de fármacos e no monitoramento terapêutico (MADHURI *et al.*, 2024).

Enquanto sistemas de detecção menos sensíveis, baseiam-se em propriedades ópticas e enfrentam limitações de resolução e interferência da matriz, o UPLC-MS/MS utiliza

analisadores de massa para separar íons com base em sua razão massa-carga, oferecendo especificidade e limites de detecção superiores (PITT, 2009; MADHURI *et al.*, 2024).

A validação de métodos analíticos é um processo fundamental de comprovação experimental que assegura a confiabilidade, a precisão e a adequação de um protocolo à sua finalidade laboratorial específica. Este procedimento é norteado por diretrizes de agências reguladoras, como a Agência Nacional de Vigilância Sanitária (ANVISA) e o Conselho Internacional de Harmonização de Requisitos Técnicos para Fármacos de Uso Humano (ICH), exigindo a avaliação de parâmetros rigorosos como seletividade, linearidade, exatidão e limites de quantificação para garantir que o método atenda às exigências das aplicações analíticas (ANVISA, 2017).

5. METODOLOGIA

A presente pesquisa foi estruturada em duas etapas experimentais complementares, conduzidas sob aprovação do Comitê de Ética no Uso de Animais (CEUA/UFERSA nº 07/2023), utilizando cães adultos, hígidos e sem raça definida (SRD). O delineamento experimental consistiu, primeiramente, na caracterização farmacocinética da dipirona isolada ($25 \text{ mg} \cdot \text{kg}^{-1}$, intravenosa, 11 animais) e, em um segundo momento, estudo da associação farmacocinética entre a dipirona e o tramadol ($2 \text{ mg} \cdot \text{kg}^{-1}$, intravenosa, 9 animais) através de modelo crossover. A redução no número de animais entre as etapas ocorreu devido a inconsistências estatísticas, visando assegurar a robustez e a homogeneidade dos dados comparativos. Os animais foram controle deles mesmos e o período entre a administração dos tratamentos (*washout*) foi de 15 dias. O intervalo de *washout* entre os tratamentos foi estabelecido de forma a garantir a depuração biológica completa dos fármacos e seus metabólitos, levando em conta o tempo de meia-vida das drogas utilizadas.

A logística de coleta de dados foi padronizada para ambos os estudos, compreendendo 16 coletas de sangue seriadas ao longo de 48 horas para cada animal. O rigor analítico foi garantido pelo uso da cromatografia líquida de ultraeficiência acoplada à espectrometria de massas (UPLC-MS/MS), para detecção de todos os metabólitos. Todo o método foi rigorosamente validado conforme as diretrizes da RDC 166/2017 da ANVISA e do ICH, assegurando seletividade, linearidade e estabilidade das amostras. Os metabólitos M2 e M5 do tramadol foram analisados por equivalentes de M1, pois os padrões analíticos de ambos

não estão disponíveis para uso e devido à estrutura química dos metabólitos serem similares, é um método possível de ser realizado.

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CAPÍTULO 2: Artigo - Farmacocinética da Dipirona**TÍTULO**

Pharmacokinetic Profile of Two Active Dipyrone Metabolites, 4-Methylaminoantipyrine (MAA) and 4-Aminoantipyrine (AA), Following Intravenous Administration in Dogs: A Preliminary Study

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Pharmacokinetic Profile of Two Active Dipyrone Metabolites, 4-Methylaminoantipyrine (MAA) and 4-Aminoantipyrine (AA), Following Intravenous Administration In Dogs: A Preliminary Study

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Simple Summary: Dipyrone is widely used to control pain. However, although the standard dose for routine use is 25 mg·kg⁻¹, no studies addressing the pharmacokinetics of this drug employing ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) are available to date. Thus, the aim of this study was to determine the pharmacokinetic profile of the active metabolites of dipyrone 4-methylaminoantipyrine (MAA) and 4-aminoantipyrine (AA) administered intravenously, at 25 mg·kg⁻¹, in eleven mixed-breed dogs (weighing 14.43 ± 2.86 kg). Serial blood samples were collected after the drug administration, stored at -80 °C and analyzed by high-performance chromatography coupled to mass spectrometry. A Principal Component Analysis (PCA) indicated two groups of metabolizers, fast and slow, for MAA. This demonstrates metabolism variability within the same group of mixed-breed dogs. Furthermore, the minimum effective concentrations for promoting analgesia in humans were reached for dipyrone metabolites. These results suggest that dipyrone achieves therapeutic plasma concentrations with minimal adverse effects. However, further pharmacogenetic and pharmacotherapeutic studies are required to refine dosing recommendations.

Abstract: This study aimed to determine the pharmacokinetic profile of the active dipyrone metabolites, 4-methylaminoantipyrine and 4-aminoantipyrine, following intravenous administration in dogs. Eleven mixed-breed dogs received a 25 mg·kg⁻¹ dipyrone dose and blood

samples were collected at 0, 5, 15, 30 and 45 min, as well as at 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h. Plasma concentrations of both metabolites were analyzed by ultra-performance liquid chromatography coupled to mass spectrometry. The PKSolver 2.0 and GraphPad Prisma 10 software programs were used for pharmacokinetic and statistical analyses, applying a Principal Component Analysis for MAA and descriptive statistics for both metabolites. Two groups were noteworthy concerning MAA: slow metabolizers (SM) and normal/rapid metabolizers (NM). Significant differences were observed between half-life ($T_{1/2}$) and MRT_{0_info} values between the MAA groups. The $T_{1/2}$ and MRT_{0_info} were 44.44 ± 11.74 and 32.62 ± 16.53 h for the SM group and 11.25 ± 5.37 and 7.44 ± 4.25 h for the NM group, respectively. The C_{max} of AA was 2.80 ± 1.43 $\mu\text{g mL}^{-1}$. Metabolites were detectable for 48 h in all animals for MAA and seven for AA. These findings suggest that metamizole reaches analgesia plasma concentrations associated with cyclooxygenase inhibition with few adverse effects in dogs. However, additional pharmacogenetic and pharmacotherapeutic monitoring studies are required.

Keywords: analgesia; chromatography; metamizole; metabolism.

1. Introduction

Pain control in animals is of utmost importance in clinical practice, promoting physical condition and quality of life improvements, both of which are essential for their well-being [1]. Dogs are constantly subjected to surgical procedures or treated for pathological conditions that culminate in pain, requiring adequate pain control [2]. Thus, the use of analgesic drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and non-opioid analgesics represents an important practice for treating acute pain in small animals [3].

Metamizole, more commonly known as dipyrone, is an analgesic indicated for pain treatment in both humans and animals [4]. Although previously classified as an NSAID due to non-selective inhibition of cyclooxygenase (COX), with emphasis on COX-3 and displaying weak COX-1 and COX-2 inhibition, metamizole exhibits weak anti-inflammatory action and promotes the activation of opioidergic pathways, thus being currently classified as a non-opioid analgesic [5,6]. However, its use is prohibited in countries such as the United States, Japan and Australia due to side effects like agranulocytosis and aplastic anemia in humans, although several countries in Europe, Asia and South America, including Brazil, continue to market this drug [6,7].

Pharmacokinetics, defined as the study of drug absorption, distribution, metabolism, and excretion (ADME), is essential for understanding drug behavior in both physiological and pathological

conditions. Studies in this regard are useful for appraising certain parameters, such as dosage regimen and drug behavior following administration [8]. Despite restrictions on the use of dipyrone in many countries, pharmacokinetic studies concerning this drug in different animal species are paramount for evaluating applied doses, administration intervals and plasma concentrations [9–14].

Metamizole is a prodrug rapidly hydrolyzed in plasma to its main metabolite, 4-methylaminoantipyrine (MAA) [15]. This metabolite is, in turn, metabolized by cytochrome P450 (CYP) enzymes in the liver into 4-formylaminoantipyrine (FAA) through oxidation processes and into 4-aminoantipyrine (AA) by demethylation [16]. Subsequently, AA can be acetylated to 4-acetylaminoantipyrine (AAA) by N-acetyltransferase 2 (NAT2) [15,17]. The analgesic character of this drug is mainly attributed to its two active metabolites, MAA and AA [18].

Regarding research on dipyrone pharmacokinetics in dogs, Kalchofner Guerrero et al. [19] described the plasma concentrations of active metamizole metabolites in relation to time via oral administration in dogs, but did not describe their complete pharmacokinetic profiles. Only Giorgi et al. [9] reported this, for female Labradors, in which different administration routes of a 25 mg kg⁻¹ dose, including by the intravenous route, were evaluated using high-performance liquid chromatography (HPLC) coupled to an ultraviolet detector (HPLC-UV).

In this context, this study aimed to evaluate the pharmacokinetic profiles of two active dipyrone metabolites (MAA and AA) administered intravenously at 25 mg kg⁻¹ in male and female mixed-breed dogs through ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS). In comparison, Giorgi et al. [9] assessed only a single breed, detecting interindividual metabolism changes. Furthermore, the use of liquid chromatography coupled with mass spectrometry (LC-MS) offers significant advantages over the HPLC-UV method, including greater metabolite detection and quantification sensitivity and specificity, allowing for more accurate and detailed pharmacokinetic profile assessments.

2. Material and Methods

2.1. Animals and study preparation

This study was approved by the Federal Rural Semi-Arid University Animal Use Ethics Committee (CEUA/UFERSA), under number 07/2023 (2 May 2023). Eleven adult dogs (five females and six males), with a minimum age of 1 year and a maximum of 5 years (1.72 ± 1.35), intact and healthy, weighing 14.43 ± 2.86 kg, all mixed breeds, were obtained from a non-governmental organization (NGO), located in Caraúbas, Rio Grande do Norte, Brazil. The consent and authorization form to carry out the study was signed by the people responsible for the animals.

The dogs were grouped in kennels on the premises of UFERSA's Internal Veterinary Medicine Laboratory and subjected to 8-h food and 4-h water fasts. All animals underwent physical and laboratory examinations (complete blood counts and serum biochemistry urea, creatinine, total protein, albumin, alanine aminotransferase and alkaline phosphatase) and were included in the study after being certified as healthy.

The 11 animals were manually restrained, and the cephalic vein region underwent trichotomy and antisepsis for insertion of a 20 G catheter, coupled to a 3-way stopcock to maintain viable venous access. All animals were subjected to the same dipyrone treatment (D-500®, Zoetis, São Paulo, Brazil) administered intravenously at 25 mg·kg⁻¹ for 2 min using an infusion pump (DigiPUMP SR 31X-Digicare®, Rio de Janeiro, Brazil).

All animals were evaluated for any sign of behavioral changes and adverse effects, such as salivation, sweating, muscle spasms, restlessness and tachypnea, assessing symptom onset and duration.

2.2. Pharmacokinetic study

To determine plasma MAA and AA concentrations, 4 mL of blood was collected from the cephalic vein of all animals, at the following time points: 0 (prior to drug administration) and 5, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h. The samples were stored in tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 3500 rpm for 10 min to obtain blood plasma, which was then stored in cryogenic tubes at -80°C, for later analysis by UPLC-MS/MS.

2.3. Sample extraction procedures

Aliquots of the obtained plasma samples (250 µL) were mixed with 10 µL of a 0.1 mg mL⁻¹ metoprolol solution (internal standard) and 800 µL of acetonitrile, followed by vortexing for 60 seconds and centrifugation for 10 min at 10,000 rpm. The supernatants (900 µL) were transferred to vials and 5 µL were injected into the UPLC-MS/MS chromatographic system, consisting of a Nexera 2 UPLC coupled to an LCMS-8040 mass spectrometry detector (Shimadzu, Japan) and a Phenomenex UPLC Luna Omega C18 column (1.6 µm, 2.1 × 50 mm) (Phenomenex, Torrance, CA, USA).

2.4. Analytical UPLC-MS/MS Conditions

The mobile phase comprised acetonitrile and a 0.1% formic acid solution (75:25, v/v) at 0.3 mL min⁻¹. The run time was set at 2.0 min and the injected sample volume, 5.0 µL. The column temperature was adjusted to 40 °C, and the autosampler refrigerator was set to 5 °C.

The mass spectrometer was set to multiple reaction monitoring (MRM) mode in positive ionization mode (ESI+). The applied collision energy and cone voltage were 28 and 25 V, respectively. The nebulizer gas

flow rate was 3 L min⁻¹, using argon as the collision gas at a flow rate of 15 L min⁻¹. The mass spectrometer was set to monitor the band transition of the parent and daughter ions. A dwell time of 0.1 s was established, and MRM data were acquired and analyzed using the Labsolution software version 5.114 (Shimadzu, Kyoto, Japan).

The mass and charge ratio (*m/z*) of the main analyzed ions were MAA (218.1), AA (204.1) and metoprolol (268.2), and for the daughter ions, MAA (159.1), AA (77.00) and metoprolol (131.0).

2.5. Validation

The analytical method was revalidated according to the International Conference on Harmonization ICH (2018) and the National Health Surveillance Agency [20] criteria. A drug-free plasma was spiked with a standard solution to prepare a calibration curve. Quality control samples (spots) were prepared at low (80 ng mL⁻¹), medium (5600 ng mL⁻¹) and high (40,000 ng mL⁻¹) levels for both MAA and AA and used to determine absolute recoveries and intra- and inter-day precisions. Selectivity was assessed by preparing a curve using the lower limit of quantification (LLOQ), established as 0.076 µg mL⁻¹ for both metabolites in drug-free plasma. Stability concerning the biological matrix at -70 °C, bench temperature at room temperature (20 °C) and samples were assessed using an autosampler.

2.6. Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated by applying a non-compartmental model with PKSolver 2.0 software. The observed variables were as follows: maximum plasma concentration (*C*_{max}), plasma concentration at time zero (*C*₀), time to reach *C*_{max} (*T*_{max}), half life (*T*_{1/2}), area under the plasma concentration curve from time zero to the time of the last measurable concentration (*AUC*_{0→t}) and the extrapolation of *AUC* to infinity (*AUC*_{0→∞}), the ratio between *AUC*_{0→t} and *AUC*_{0→∞}, the area under the curve from the first time of zero to the last measurable concentration *AUMC*_{0→t}; the mean residual time from moment zero to infinity (*MRT*_{0→inf}), volume of distribution (*V*_d), which is the estimated volume of distribution based on the *AUC*, the volume of distribution at steady state (*V*_{ss}), the clearance (*Cl*), the terminal phase rate constant (*λ*_z).

2.7. Statistical Analyses

Descriptive statistical analyses were performed using Python (version 3.10). Data were initially tested for normality using the Kolmogorov–Smirnov test. Comparative statistics were performed applying an Analysis of Variance (ANOVA) with a post hoc Tukey test and Mann–Whitney between groups for MAA data (SL, NM and all dogs), with significance set at $p < 0.05$. A post hoc t-test for independent samples was performed to calculate Cohen’s d and statistical power, assessing the magnitude and reliability of differences between metabolizer groups (SL and MN groups). In addition, a Principal Component Analysis (PCA) was performed for MAA pharmacokinetic variables. Data were presented as means $\pm$ standard deviations for AA and MAA, and the median (maximum–minimum) was also presented for MAA.

3. Results

The method was revalidated to ensure data accuracy according to other studies [14, 21,22]. All calibration curves presented R^2 greater than 0.99, demonstrating linearity between equipment responses and curve concentrations. The method also presented good selectivity, reproducibility and repeatability, with relative standard deviations below 5%. The samples were stable under the analysis conditions.

After intravenous metamizole administration, both MAA and AA metabolites were analyzed for 48 h in all dogs, with MAA quantified at this time in all animals, while AA was quantified in seven. The plasma concentrations of both metabolites over time are described in Figures 1 and 2.

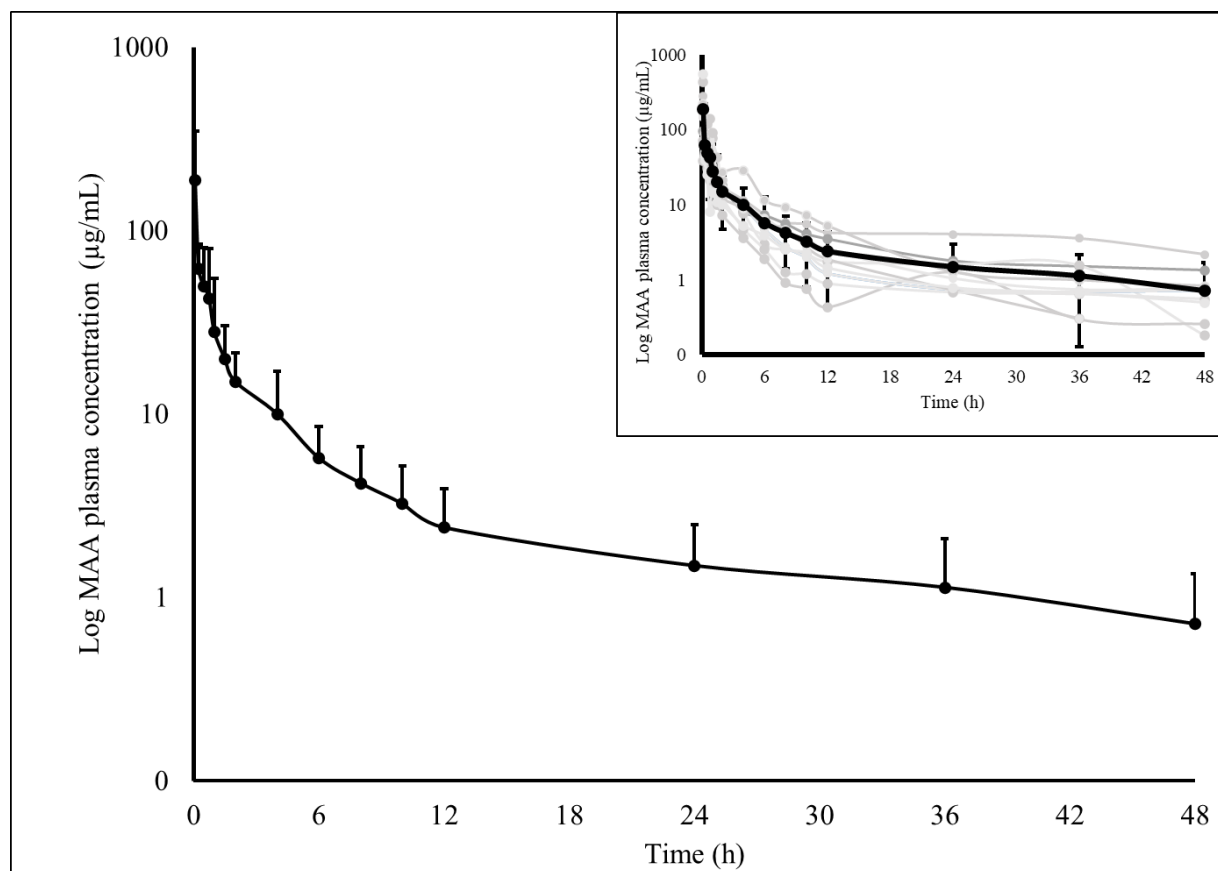


Figure 1. Log of the plasma concentration of 4-methylaminoantipyrine (MAA) as a function of time, at 25 mg kg^{-1} metamizole administered intravenously in dogs. The individual pharmacokinetic profiles of all animals are highlighted (grey lines). The MAA results are expressed as means and standard deviations (black lines) ($n = 11$).

Regarding general pharmacokinetic parameters, the data are expressed as the means $\pm$ standard deviations (Table 1) and compared to the results reported by Giorgi et al. [9] for dogs. All animals showed optimal correlations in the non-compartmental pharmacokinetic model employing the applied dose ($R^2 > 0.7$).

A Principal Component Analysis (PCA) was performed (Figure 3), demonstrating the correlation between the different pharmacokinetic parameters in relation to MAA (C_{max} , $T_{1/2}$, $\text{MRT}_{0-\text{inf_obs}}$, AUC_{0-t} , V_d , Cl_obs). This analysis indicated two groups of animals: the NM group ($n = 6$) represented in red (normal/fast metabolizers), and the SM group ($n = 5$) represented in blue (slow metabolizers).

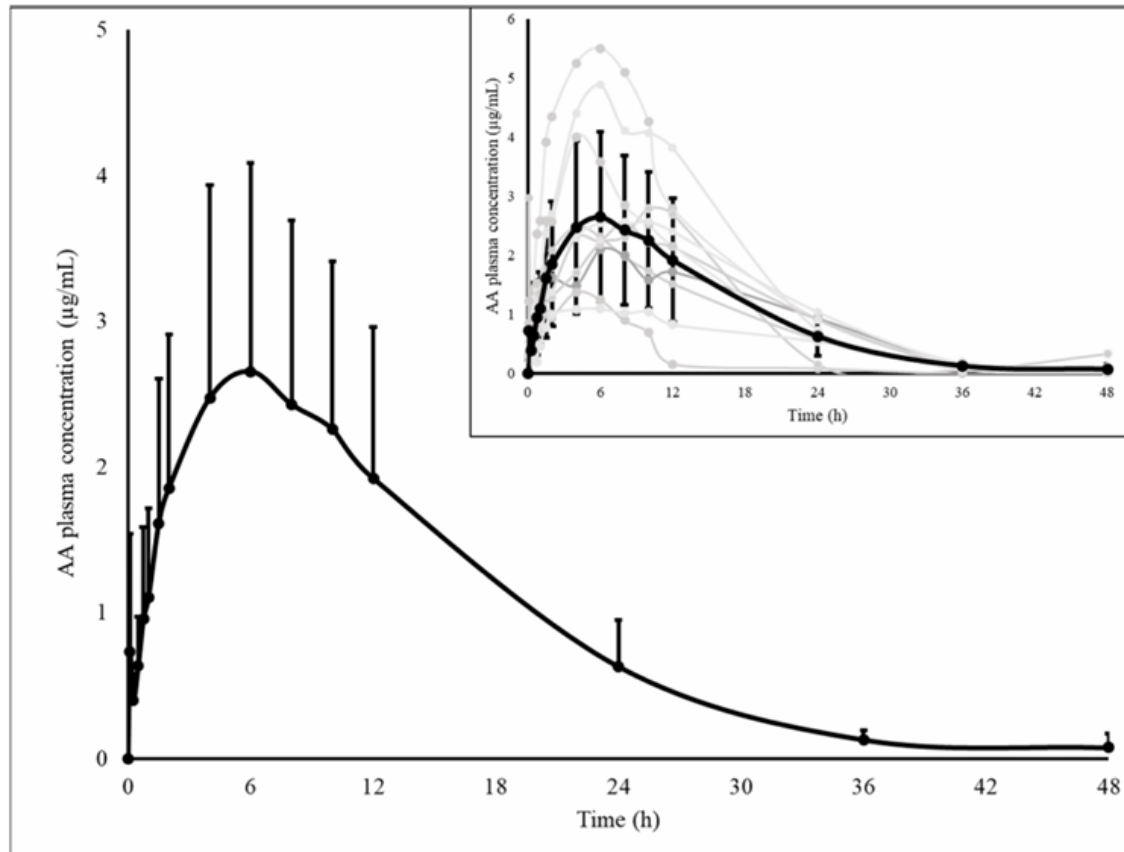


Figure 2. Plasma concentration of 4-aminoantipyrine (AA) as a function of time, at 25 mg kg⁻¹ metamizole administered intravenously. The individual pharmacokinetic profiles of all animals are highlighted (grey lines). The AA results are expressed as means and standard deviations (black lines) (n = 11).

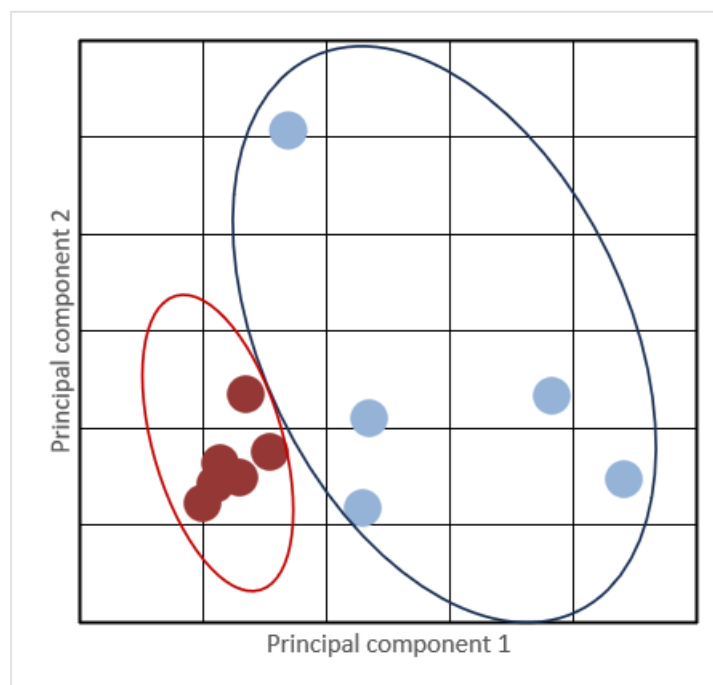


Figure 3. Principal Component Analysis (PCA) was performed for MAA, assessing the investigated pharmacokinetic parameters (C_{max} , $T_{1/2}$, MRT_{0-inf_obs} , AUC_{0-t} , V_d , Cl). Normal/fast metabolism pattern (NM group; red) and slow metabolism (SM group; blue).

Table 1. Pharmacokinetic parameters of 4-Methylaminoantipyrine (MAA) and 4-Aminoantipyrine (AA) following intravenous metamizole administration at 25 mg kg^{-1} in dogs ($n = 11$) compared to the study carried out by Giorgi et al. [9].

Parameter	Unit	MAA	MAA (Giorgi <i>et al.</i> , 2018) [9]	AA	AA (Giorgi <i>et al.</i> , 2018) [9]
		Means $\pm$ SD	Means $\pm$ SD	Means $\pm$ SD	Means $\pm$ SD
Lambda_z	1/h	0.06 $\pm$ 0.08	0.13 $\pm$ 0.04	0.11 $\pm$ 0.03	0.09 $\pm$ 0.03
T ½	h	26.39 $\pm$ 19.29	5.94 $\pm$ 2.54	6.72 $\pm$ 1.66	8.05 $\pm$ 2.56
Cmax	µg/ml	203.68 $\pm$ 159.24	21.80 $\pm$ 2.45	2.80 $\pm$ 1.43	1.29 $\pm$ 0.21
C0	µg/ml	409.23 $\pm$ 524.72	-	-	-
Tmax	h	-	-	6.18 $\pm$ 2.27	5.33 $\pm$ 1.63
AUC 0-t	µg/ml*h	205.71 $\pm$ 108.18	45.34 $\pm$ 9.64	43.87 $\pm$ 18.94	17.97 $\pm$ 2.91
AUC 0-inf_obs	µg/ml*h	240.02 $\pm$ 127.38	46.79 $\pm$ 9.15	-	21.08 $\pm$ 4.34
AUC 0-t/0-inf_obs		0.88 $\pm$ 0.12	-	-	-
AUMC 0-inf_obs	µg/ml*h ²	5164.94 $\pm$ 5735.48	246.57 $\pm$ 31.01	540.32 $\pm$ 245.01	273.97 $\pm$ 117.74
MRT 0-inf_obs	h	18.88 $\pm$ 17.07	5.37 $\pm$ 0.81	12.09 $\pm$ 2.79	12.62 $\pm$ 3.13
Vd	(L/kg)	4.38 $\pm$ 3.62	4.95 $\pm$ 0.99	-	-
Vss	(L/kg)	-	-	6.72 $\pm$ 3.74	13.85 $\pm$ 3.68
Cl	(ml/h/kg)	147.67 $\pm$ 103.85	552.43 $\pm$ 98.34	712.76 $\pm$ 45.23	1.224.03 $\pm$ 228.74

Legend: Lambda_z: terminal phase rate constant; T1/2 : half-life; CMax: maximum plasma concentration; C0: plasma concentration at time zero; Tmax: time to reach maximum plasma concentration; CMax:: maximum plasma concentration; AUC0→t: area under the curve from 0 to the last measurement; AUC0→∞: area under the curve from 0 to infinity; AUC0→t/AUC0→∞: the ratio between AUC0→t and AUC0→∞; AUMC0→t: area under the curve from the first moment from zero to the last measurable concentration; MRT0→∞: mean residual time from 0 to infinity; Vd: apparent volume of distribution; Vss: volume of distribution at steady state; Cl: total body clearance.

Table 2 depicts MAA metabolism differences among the analyzed animals. Five animals (two males and three females) presented a slow metabolism pattern (SM group), while six (three males and three females) presented a normal/fast metabolism pattern (NM group), demonstrated mainly by significant differences in T½, and MRT0-inf_obs values. The data are expressed as means $\pm$ standard deviations and medians (maximum - minimum). Effect size and power analyses confirmed the

robustness of statistically significant differences for $T_{1/2}$, MRT_{0-inf_obs} , and $AUC_{0-t}/AUC_{0-inf_obs}$ between metabolizer groups. These parameters showed very large effect sizes (Cohen's $d > 2.0$) and high statistical power ($>88\%$).

Regarding behavioral and adverse effects, three animals presented tachypnea, two belonging to the SM group and one to the NM group, at the beginning of the drug administration, with an average duration of 15 min.

Table 2 - Pharmacokinetic parameters determined for 4-Methylaminoantipyrine (MAA) following intravenous metamizole administration at 25 mg kg⁻¹ in dogs (n = 11), all of mixed breeds, with metabolism differences detected among individuals, categorized as slow metabolizers – ML (n = 5, two males and three females) and normal/rapid

metabolizers – MNR (n = 6, three males and three females).

MAA		All Dogs (n = 11)	Slow Metabolized (SL) (n = 5)		Normal/Quick Metabolized (NM) (n = 6)	
Parameter	Unit	Means ± SD	Means ± SD	Median (Max–Min)	Means ± SD	Median (Max–Min)
λ_{z_z}	1/h	0.06 ± 0.08	0.02 ± 0.004	0.017 (0.020–0.011)	0.09 ± 0.09	0.05 (0.29–0.04)
$T_{1/2}$	h	26.39 ± 19.29	44.44 ± 11.74 *	38 (58.35–33.57) *	11.25 ± 5.37	12.58 (16.8–2.35)
C_{max}	µg/mL	203.68 ± 159.24	238.29 ± 197.92	223.68 (548.74–44.13)	174.84 ± 131.07	149.14 (428.98–71.78)
C_0	µg/mL	409.23 ± 524.72	606.69 ± 757.10	434.48 (1912.98–38.00)	244.67 ± 143.60	211.38 (493.71–77.45)
T_{max}	h	-	-	-	-	-
AUC_{0-t}	µg/mL×h	205.71 ± 108.18	239.78 ± 88.95	250.42 (336.07–103.95)	177.32 ± 122.27	166.84 (404.65–67.37)
AUC_{0-inf_obs}	µg/mL×h	240.02 ± 127.38	306.76 ± 106.84 *	326.29 (441.35–148.5) *	184.41 ± 123.09	174.99 (407.78–69.99)
$AUC_{0-t/0-inf_obs}$		0.88 ± 0.12	0.78 ± 0.1 *	0.76 (0.90–0.65) *	0.95 ± 0.04	0.95 (0.99–0.90)
$AUMC_{0-inf_obs}$	µg/mL×h ²	5164.94 ± 5735.48	9733.64 ± 5762.53	6899.49 (17,240.51–3583.31)	1357.68 ± 979.22	1281.33 (2857.61–153.47)
MRT_{0-inf_obs}	h	18.88 ± 17.07	32.62 ± 16.53 *	32.43 (52.83–12.97) *	7.44 ± 4.25	7.18 (14.09–2.02)
V_d	(L/kg)	4.38 ± 3.62	6.32 ± 4.29	4.71 (13.62–2.74)	2.77 ± 2.13	2.33 (6.77–1.06)
Cl	(mg/kg)/(ug/mL)/h	0.15 ± 0.10	0.09 ± 0.04	0.07 (0.16–0.05)	0.19 ± 0.12	0.14 (0.35–0.06)

Data are presented as means ± standard deviations and medians (maximum – minimum). Legend: λ_{z_z} : terminal phase rate constant; $T_{1/2}$: half-life; C_{max} : maximum plasma concentration; C_0 : plasma concentration at time zero; T_{max} : time to reach maximum plasma concentration; C_{max} : maximum plasma concentration; AUC_{0-t} : area under the curve from 0 to the last measurement; AUC_{0-inf} : area under the curve from 0 to infinity; AUC_{0-t}/AUC_{0-inf} : the ratio between AUC_{0-t} and AUC_{0-inf} ; $AUMC_{0-t}$: area under the curve from the first moment from zero to the last measurable concentration; MRT_{0-inf} : mean residual time from 0 to infinity; V_d : apparent volume of distribution; V_{ss} : volume of distribution at steady state; Cl : total body clearance.

*Indicates statistical difference between the SL group, and NM group/all dogs ($p < 0.05$).

4. Discussion

This is a first-time report concerning the evaluation of active dipyrone metabolite pharmacokinetics in male and female dogs employing the UPLC-MS method. Although one study describing pharmacokinetic parameters by different routes, including intravenous

administration in dogs, has been carried out by Giorgi et al. [9], important differences in their sampling design and applied analytical method compared to the present study are noteworthy. A 25mgkg^{-1} dose was chosen due to its routine use in dogs in clinical practice. One study carried out in the postoperative period of ovariohysterectomy (OH) surgery in bitches assessed the analgesic effects and physiological and hematological parameters of 15, 25 and 35 mg kg^{-1} dipyrone doses administered intravenously [23]. The 25 and 35 mgkg^{-1} doses were efficient in promoting analgesia with no hematological and clinical parameter changes, while the 15 mg kg^{-1} dose did not promote satisfactory postoperative analgesia. The authors of that study also emphasized that, since no pain score increases were observed up to 8 h after administration, it is likely that metamizole administered in this range may be able to provide adequate analgesia.

In another clinical study, the administration of dipyrone alone at 25 mg kg^{-1} led to a higher degree of analgesia in bitches undergoing the same OH procedure, as well as a reduced need for rescue analgesics during the postoperative period [24]. These findings reinforce the choice of the 25 mg kg^{-1} dose in the present study and suggest that dipyrone exhibits adequate clinical efficacy when applied in animals after painful stimulation, although pharmacokinetic parameter investigations are necessary in this sense.

Table 1 presents significant differences in the pharmacokinetic parameters of the metabolites MAA and AA when compared to the study by Giorgi et al. (2018) [9], which can largely be attributed to the greater interindividual variability resulting from the inclusion of both sex and multiple dog breeds in the present study, as opposed to the use of a single breed and only female in the previous research [9,24]. This diversity led to greater data dispersion, as evidenced by the wide variation in MAA half-life ($T_{1/2}$), which reached $26.39 \pm 19.29\text{ h}$ compared to $5.94 \pm 2.54\text{ h}$ reported by Giorgi, as well as significantly higher values of C_{max} ($203.68 \pm 159.24\text{ }\mu\text{g/mL}$ vs. $21.80 \pm 2.45\text{ }\mu\text{g/mL}$) and AUC_{0-t} ($205.71 \pm 108.18\text{ }\mu\text{g/mL}\cdot\text{h}$ vs. $45.34 \pm 9.64\text{ }\mu\text{g/mL}\cdot\text{h}$). For AA, although the $T_{1/2}$ values were similar ($6.72 \pm 1.66\text{ h}$ vs. $8.05 \pm 2.56\text{ h}$), the C_{max} was more than twice as high ($2.80 \pm 1.43\text{ }\mu\text{g/mL}$ vs. $1.29 \pm 0.21\text{ }\mu\text{g/mL}$), and the AUC_{0-t} was also considerably greater ($43.87 \pm 18.94\text{ }\mu\text{g/mL}\cdot\text{h}$ vs. $17.97 \pm 2.91\text{ }\mu\text{g/mL}\cdot\text{h}$). The lower clearance observed for MAA ($147.67 \pm 103.85\text{ mL/h/kg}$ vs. $552.43 \pm 98.34\text{ mL/h/kg}$) and the increased apparent clearance for AA ($712.76 \pm 45.23\text{ mL/h/kg}$ vs. $1224.03 \pm 228.74\text{ mL/h/kg}$) further reflect this inter-animal heterogeneity. These findings highlight the relevance of inter-individual, particularly genetic and physiological, variability in pharmacokinetic studies and reinforce the importance of including different breeds in more representative preclinical assessments.

The minimum MAA concentrations to promote 50% cyclooxygenase enzyme inhibition (IC_{50} – COX-1 and COX-2) have been described in humans, comprising an important tool used to determine drug potency, with values of $0.553\text{ }\mu\text{g mL}^{-1}$ for IC_{50} - COX1 and $0.926\text{ }\mu\text{g mL}^{-1}$ for IC_{50} -

COX2 [25,26]. This indicates that achieved plasma concentrations promoted minimal COX-1 and COX-2 inhibition in both Giorgi et al. [9] and in the present study, suggesting that the main active dipyrone metabolite presents high inhibitory effects against these enzymes. COX-1 and COX-2 are two isoenzymes, the former considered constitutive and physiological, while the latter is inducible [27]. These evaluations comprise assessments on analgesic dipyrone mechanisms of action, as this agent presents a complex inhibition mechanism regarding COX isoenzymes (1, 2 and 3), although it also acts quickly on opioid receptors and on the cannabinoid system [5,28].

A study comparing a 25 mg kg⁻¹ dipyrone dose every 24 h and 12.5 mg kg⁻¹ every 12 h in cats undergoing OH demonstrated COX-1 and COX-2 inhibition at both doses, with equally effective analgesia promotion [29]. These data reinforce the need to evaluate the applied dose regimen, supporting metamizole metabolism differences between dogs and cats.

Regarding pharmacokinetic parameters, the T_{1/2} value observed for MAA in this study was considerably higher (26.39 h) (Table 1) than those previously reported for dogs (5.94 h) [9], cats (4.42 h) [10], and donkeys (3.62 h) [14], at the same dose by the same administration route. It is important to note that the MAA detection and quantification carried out herein were noted for up to 48 hours, which also contributed to the increase in the average T_{1/2} of the analyzed animals. These findings may be associated to differences between species and individuals, as well as in enzymatic expression, and analytical method sensitivity, since mass spectrometry, a very sophisticated method with high selectivity and sensitivity for analyte analysis and separation [30], was employed herein. A direct comparison with the findings reported by Giorgi et al. [9] for dogs highlights sex, breed and size differences, as, in the aforementioned study, female Labradors averaging 36 to 42 kg were analyzed, in contrast to the present study, in which both male and female dogs of mixed breeds averaging 14.43 kg were used.

Human studies suggest differences in the activity of some CYP enzymes, such as CYP3A4, which is responsible for over 50% of the metabolism of commonly used drugs (including metamizole), between genders. In some studies, greater CYP3A4 activity has been detected in women compared to men, although some in vitro data are used, while in other studies, no significant differences related to CYP3A4 activity at the gastrointestinal level were suggested between men and women [31]. Studies on dog pharmacogenetics report pharmacokinetics and pharmacodynamics differences between breeds, so the same treatment used in different breeds may result in different findings [32].

Some animals presented slow metabolism (SM) behavior, which increased T_{1/2} (44.44 h), MRT (32.62 h), C_{max} (238.29 µg mL⁻¹) and V_d (6.32 mL kg⁻¹) values compared to normal/rapid metabolizers (NM), with values of 11.25 h, 7.44 h, 174.84 µg mL⁻¹ and 2.77 mL kg⁻¹, respectively. In humans, CYP3A4, CYP2B6, CYP2C8 and CYP2C9 are mainly responsible for MAA N-demethylation, transforming this compound into AA [15,16],

while oxidation of MAA to FAA is carried out by nonspecific CYP enzymes.

In dogs, this may justify the metabolism differences observed herein, as mixed breed animals do not present a specific pattern, resulting in potential differences in the expression of different CYP genes, leading to fast or slow metabolization profiles. These data were also suggested by Fux et al. [33] when applying 40 mg kg⁻¹ metamizole in eight calves 10 minutes prior to general anesthesia for umbilical surgery, reporting that five animals showed faster MAA to AA metabolization. The authors discuss individual differences in metabolic hepatic activity and CYPs, which present interindividual expression and activity variabilities in cattle [34]. However, it is important note that Fux et al. [33] analyzed animals also administered other drugs, such as meloxicam, ketamine and xylazine, which can lead to metabolism variations. These differences between CYP activities may, thus, justify the metabolism differences observed herein between individuals, as metamizole metabolism is dependent on these enzymes through N-demethylation and C-oxidation (MAA to FAA) (Figure 4).

The detected plasma AA concentrations in this study were lower than MAA concentrations, similar to findings reported for cats [10,35], dogs [9] and horses [12] administered the same dose, and for donkeys administered different doses [14]. This suggests that the MAA to AA metabolism occurs similarly in different animal species, *i.e.*, the production of AA and the conversion of AA into AAA does not present variable metabolism profiles between species or individuals, due to low AA concentrations. The AUC was also lower, as AA is a product of MAA metabolism [15].

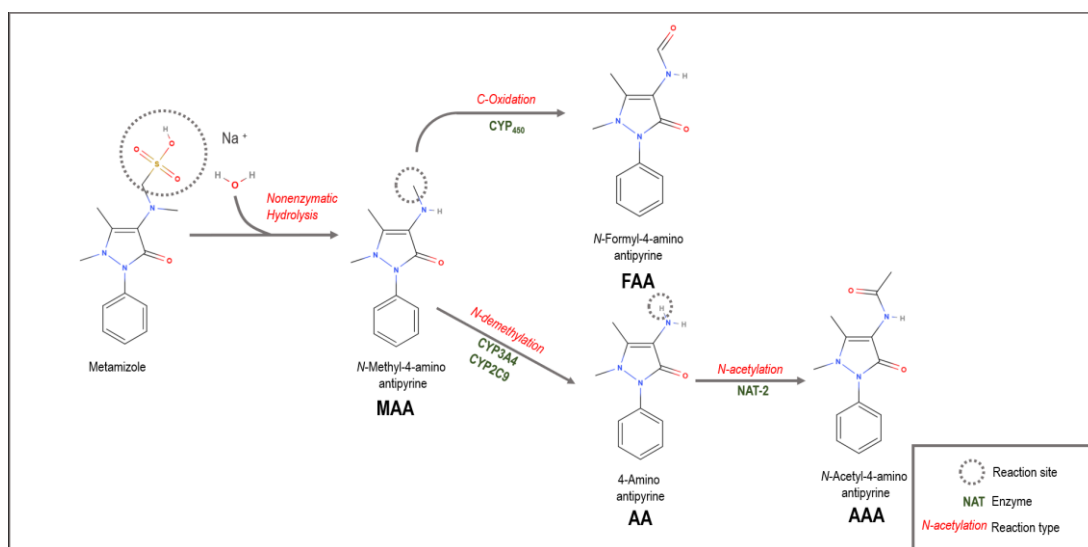


Figure 4. Metamizole metabolism.

The plasma concentration of MAA was rapidly reached, due to the abrupt entry of metamizole into the vascular compartment, which is quickly hydrolyzed into MAA. The MAA C_0 value was considerably higher in this study ($456.93 \mu\text{g mL}^{-1}$) compared to the C_{max} value reported by Giorgi et al. [9]. These differences reflect the employed analytical method, and the physical and genetic characteristics of the animals assessed herein. The MAA AUC was quite high, above other studies, representing high drug exposure over time in the present study.

Regarding pharmacokinetic AA data, the $T_{1/2}$ value reported in the present study was of 6.72 h, comparable to dogs (8.05 h) as reported by Giorgi et al. [9], as well as donkeys (7.11 h) [14] and cats (13.66 h) as reported by de Paula et al. [10], reinforcing a slower metamizole metabolism in felines. The analyzed AA parameters differed only slightly from data previously reported for dogs, demonstrating similar AA metabolism profiles in both studies carried out on dogs and suggesting that canine metabolic routes do not vary significantly. MAA and AA pharmacokinetic and metabolism profiles are quite distinct, with AA following a model similar to the intramuscular administration profile, unlike MAA, which follows an intravenous administration profile. This is due to the transformation of MAA into AA. In this sense, Arcoverde et al. [21] carried out pharmacotherapeutic monitoring of dipyrone in donkeys, reporting AA accumulation during dipyrone administration carried out every 12 h. These data should be assessed in dogs to verify whether the same is observed in canines and evaluate the clinical implications.

Regarding adverse effects, episodes of vomiting and salivation were not observed in dogs, although they have been reported in cats [10,35], with the only effect observed herein encompassing increased respiratory rates, lasting an average of 15 min in three animals, two from the SM group and one from the NM group. It is important to highlight that these animals presented agitated behavior, which may have contributed to the occurrence of this effect and is not clinically relevant. No donkeys [14] or horses [12] presented adverse effects. The clinical studies carried out by Imagawa et al. [23] in dogs and Pereira et al. [29] in cats did not report relevant adverse effects regarding hematological changes, while Zanuzzo et al. [24] detected transient platelet aggregation inhibition. This is noteworthy because dipyrone is banned in several countries due to the hematological effects found in humans [16]. Thus, further studies correlating metamizole pharmacokinetic profiles with physiological and hematological changes should be carried out, as well as more studies employing different doses and administration intervals to determine alternatives for different metamizole doses in dogs.

5. Conclusions

The findings reported herein suggest that achieved plasma concentrations may promote analgesic and anti-inflammatory effects associated to cyclooxygenase inhibition with few adverse effects in dogs. Two active dipyrone metabolites were formed in dogs, and the analytical UPLC-MS method was deemed adequate for their detection. Further studies involving pharmacokinetics, pharmacogenetics (concerning the polymorphism associated with dipyrone metabolism) and pharmacotherapeutic monitoring are necessary to determine $T_{1/2}$ differences in order to define an appropriate dipyrone dose range for dogs.

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Institutional Review Board Statement: The animal study protocol was approved by the Federal Rural Semi-Arid University Animal Use Ethics Committee (CEUA/UFERSA), under number 07/2023 (2 May, 2023).

Informed Consent Statement: Written informed consent was obtained from all the owners of the animals involved in the study.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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CAPÍTULO 3: Artigo - Farmacocinética da associação dipirona e tramadol

TÍTULO

**Influence of tramadol on the intravenous pharmacokinetics of dipyrrone active
metabolites in dogs**

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Influence of tramadol on the intravenous pharmacokinetics of dipyrrone active metabolites in dogs

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Abstract:

This study aimed to compare the pharmacokinetic profile of the active dipyrrone metabolites 4-methylaminoantipyrine (MAA) and 4-aminoantipyrine (AA) administered alone and in combination with tramadol in dogs. Nine mixed-breed dogs, weighing 15.33 ± 2.25 kg, participated in a two-treatment crossover design: G1 received intravenous dipyrrone ($25 \text{ mg} \cdot \text{kg}^{-1}$) and G2 received dipyrrone ($25 \text{ mg} \cdot \text{kg}^{-1}$) combined with tramadol ($2 \text{ mg} \cdot \text{kg}^{-1}$), with a 15-day washout. Blood samples were collected up to 48 hours and plasma concentrations analyzed using UPLC-MS/MS. Pharmacokinetic parameters were calculated using PKSolver

2.0. Normally distributed data were analyzed with the t-test, and non-normal data with the Mann–Whitney test ($p < 0.05$); parameters with significant differences were subjected to Pearson correlation. Analyses were performed using Python®. For MAA, C_{max} and C_0 differed significantly. For AA, AUC_{0-t} , $AUC_{0 \rightarrow \infty}$, Cl , and $MRT_{0 \rightarrow \infty}$ differed significantly. Tramadol and its metabolites were also described. The pharmacokinetic interaction between dipyrone and tramadol increased the bioavailability of AA and inhibited the conversion of tramadol to M1, without direct changes in the severe adverse effects associated with MAA. Further studies involving nociceptive stimuli and multiple-dose regimens are needed to assess clinical relevance.

Keywords: Metamizole; 4-Methylaminoantipyrine; 4-Aminoantipyrine; O-Desmethyltramadol; N-Desmethyltramadol.

Introduction

Metamizole, also known as dipyrone, is an analgesic compound with antipyretic and spasmolytic properties, belonging to the pyrazolone derivatives (Schütter *et al.*, 2024; Garcia del Campo *et al.*, 2021). Its mechanism of action involves interference with prostaglandin synthesis through the reduction of the oxidative state of the cyclooxygenase (COX) enzyme, acting on both COX-1 and COX-2. However, COX-3 is considered the most sensitive isoform, primarily located in the central nervous system and derived from COX-1 (Chandrasekharan *et al.*, 2002). Opioidergic pathways have also been reported as part of the drug's mechanism of action (Tortorici *et al.*, 1996; Jasińska, Maślanka and Jaroszewski, 2014).

The clinical use of dipyrone in small animals is significant in some South American countries, including Brazil. For a long time, this compound was classified as a nonsteroidal anti-inflammatory drug; however, recent classifications define it as a non-opioid analgesic. Regarding its metabolism, dipyrone is known to be a prodrug that is rapidly chemical-

hydrolyzed in plasma into its main metabolite, 4-methylaminoantipyrine (MAA) (Levy, Zylber-Katz, and Rosenkranz, 1995; Bachmann *et al.*, 2022). MAA is an active metabolite and undergoes further metabolism to form a second active metabolite, 4-aminoantipyrine (AA), via N-demethylation, and an inactive metabolite, 4-formylaminoantipyrine (FAA), via C-oxidation (Levy, Zylber-Katz; Rosenkranz, 1995; Lutz, 2019; Bachmann *et al.*, 2022). Additionally, AA may undergo acetylation to produce another inactive metabolite, 4-acetylaminoantipyrine (AAA) (Lutz, 2019). Pharmacokinetic studies investigating the use of dipyrone alone have been previously reported (Kalchofner *et al.*, 2015; Giorgi *et al.*, 2018; Mouta *et al.*, 2025).

Tramadol is an opioid analgesic widely used for pain management in humans and animals, with central nervous system activity (Subedi *et al.*, 2019; Ruel; Steagall, 2019). This drug is classified as an atypical opioid, as it produces analgesia through μ -receptor activity and exhibits a monoaminergic action by inhibiting the reuptake of norepinephrine and serotonin (Grond; Sablotzki, 2004; Bravo, Mico; Berrocso, 2017). Studies evaluating tramadol in dogs for the management of clinical and postoperative conditions are well documented in the literature (Mansfield; Beths, 2015; Donatti *et al.*, 2021).

Regarding tramadol pharmacokinetics, it is metabolized by cytochrome P450 enzymes in humans (CYP3A4 and CYP2D6) and in dogs CYP2D15 (for M1), CYP2B11, CYP3A12 e CYP2C41 (for M2). Its primary active metabolite, O-desmethyltramadol (M1), is responsible for most of its analgesic effect (Miotto *et al.*, 2017). N-desmethyltramadol (M2) is an inactive metabolite, whereas N,O-didesmethyltramadol (M5), formed from M1 metabolism, is also active (Beakley, Kaye, and Kaye, 2015; Domínguez-Oliva *et al.*, 2021).

Pharmacokinetic studies of tramadol administered alone in dogs are extensively discussed in the literature due to inconsistencies regarding the detection and minimum concentrations of M1 required to produce analgesia (Kukanich and Papich, 2004; McMillan *et al.*, 2008; Giorgi *et al.*, 2009; Giorgi *et al.*, 2010; Vettoratto *et al.*, 2010; Kukanich *et al.*, 2011).

The combination of opioid and non-opioid analgesics can produce additive effects in animals, potentially reducing adverse effects due to pharmacological synergy (Moreno-Rocha *et al.*, 2016). Guidelines from World Small Animal Veterinary Association (WSAVA, 2022) and American Animal Hospital Association (AAHA, 2022) recommend the use of multimodal analgesia to optimize pain control in dogs (Gruen *et al.*, 2022; Monteiro *et al.*, 2022). In this context, dipyrone is recognized as an important therapeutic option for the management of acute and perioperative pain. Additionally, tramadol remains cited as a pharmacological option within multimodal strategies. Its inclusion in therapeutic protocols is discussed in the guidelines, particularly in scenarios of limited availability of other controlled analgesics, allowing for the composition of diverse analgesic regimens for the canine species.

Clinical studies investigating the combination of dipyrone and tramadol in dogs have been reported for pain management in oncologic patients (Flôr *et al.*, 2013; Teixeira *et al.*, 2013). However, despite their extensive clinical use, the pharmacokinetic profile of this combination has not yet been described in dogs.

In this context, the objective of the present study was to characterize the pharmacokinetic profile of dipyrone when administered in combination with tramadol in dogs, with emphasis on the formation of its primary active metabolites, 4-methylaminoantipyrine (MAA) and 4-aminoantipyrine (AA). Additionally, the production of tramadol metabolites — O-desmethyiltramadol (M1), N-desmethyiltramadol (M2), and N,O-didesmethyiltramadol (M5) — was evaluated following intravenous administration of 25 mg·kg⁻¹ dipyrone and 2 mg·kg⁻¹ tramadol using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS). Our hypothesis is that tramadol may interfere with dipyrone hepatic metabolism through enzymatic competition, resulting in alterations in the concentrations of its active metabolites, possibly with a longer T_{1/2} of the drugs.

Materials and Methods

1.1 Animals and Study Design

The study was approved by the Animal Use Ethics Committee of the Federal Rural University of the Semi-Arid (CEUA/UFERSA), under protocol number 07/2023 (May 2, 2023). Animals were included in the study by convenience after undergoing clinical and laboratory examinations. A total of 10 animals were initially enrolled; however, one animal was excluded from statistical analysis due to inconsistencies in its results. Nine dogs (5 males and 4 females), weighing 15.33 ± 2.25 kg, healthy, intact, aged between 1 and 5 years (1.44 ± 0.88), and of mixed breed, were included. Informed consent and authorization for participation were obtained from the animals' owners.

The animals were grouped in kennels on the premises of UFERSA's Internal Veterinary Medicine Laboratory and subjected to 8-hour food and 4-hour water fasts. All dogs underwent physical examination and laboratory tests (complete blood count and serum biochemistry - urea, creatinine, total proteins, albumin, alanine aminotransferase, and alkaline phosphatase). Only dogs confirmed to be healthy were included in the study.

The nine dogs participated in a two-treatment crossover design. In group G1, they received $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone (D-500®, Zoetis, São Paulo, Brazil) intravenously, while in group G2, the same animals received $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone (D-500®, Zoetis, São Paulo, Brazil) combined with $2 \text{ mg} \cdot \text{kg}^{-1}$ tramadol (Tramadol Hydrochloride, Teuto®, Anápolis, Goiás, Brazil), also administered intravenously. The washout period between treatments was 15 days.

For drug administration, animals were manually restrained, and the cephalic vein regions underwent trichotomy and antisepsis for the insertion of two 20 G catheters, each connected to a 3-way stopcock. One access was used exclusively for drug administration, while

the other was dedicated to blood sampling to avoid cross-contamination. Drugs were administered over 2 minutes using two separate infusion pumps (DigiPUMP SR 31X - Digicare®) through the same administration venous access.

All animals were monitored for any sign of behavioral changes and adverse effects, including salivation, muscle spasms, restlessness, and tachypnea. The presence, onset, and duration of these effects were recorded.

1.2 Pharmacokinetic Study

To determine plasma concentrations of MAA, AA, tramadol, M1, M2, and M5, 4 mL of blood were collected from the cephalic vein of all animals, at the following time points: 0 (prior to drug administration), and 5, 15, 30, 45 minutes, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36, and 48 hours. Blood samples were collected through a separate venous access, which was flushed with heparinized saline solution after each collection to ensure patency. The samples were stored in tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 3500 rpm for 10 minutes to obtain plasma, which was then stored in cryogenic tubes at -80°C until analysis by ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS).

1.3 Sample Extraction Procedures

Aliquots of the obtained plasma samples (250 μL) were mixed with 10 μL of 0.1 $\text{mg}\cdot\text{mL}^{-1}$ metoprolol solution (internal standard) and 800 μL of acetonitrile, followed by vortexing homogenization for 60 seconds and centrifugation for 10 minutes at 10,000 rpm. The

supernatants (900 μL) were transferred to vials and 5 μL was injected into the UPLC-MS/MS chromatographic system, consisting of a Nexera 2 UPLC coupled to an LCMS-8040 mass spectrometer (Shimadzu, Japan) and a Phenomenex UPLC Luna Ω mega C18 column (1.6 μm , 2.1×50 mm) (Phenomenex, Torrance, CA, USA).

1.4 UPLC-MS/MS Analytical Conditions

The mobile phase consisted of acetonitrile and 0.1% formic acid solution (75:25, v/v) at $0.3 \text{ mL} \cdot \text{min}^{-1}$. The run time was set at 2.0 minutes and the injected sample volume, 5.0 μL . The column temperature was adjusted to 40°C , and the autosampler was set to 5°C . For dipyrone metabolites (MAA, AA) and tramadol with its metabolites (M1, M2, M5), the mass spectrometer was operated in multiple reaction monitoring (MRM) mode in positive electrospray ionization (ESI+). The applied collision energy and cone voltage were 28 and 25 V, respectively. The nebulizer gas flow rate was $3 \text{ L} \cdot \text{min}^{-1}$, using argon as the collision gas at a flow rate of $15 \text{ L} \cdot \text{min}^{-1}$. The mass spectrometer was set to monitor the band transition of parent and daughter ions with a dwell time of 0.1 seconds. MRM data were acquired and analyzed using the Labsolution software (Shimadzu, Japan).

The mass and charge ratio (m/z) of the main analyzed ions were: MAA (218.10), AA (204.10), tramadol (264.70), M1 (250.20), M2 (250.30), M5 (236.40), and metoprolol (268.20); while the daughter ions were: MAA (159.10), AA (77.00), tramadol (58.10), M1 (58.10), M2 (232.17), M5 (107.42), and metoprolol (131.00).

1.5 Validation

The analytical method was revalidated following the guidelines of the International Conference on Harmonization (ICH, 2018) and National Health Surveillance Agency criteria (ANVISA, 2017). A drug-free plasma was spiked with standard solution to construct a calibration curves. Quality control samples (spots) were prepared at low (1600 ng/mL), medium (5600 ng/mL), and high (40,000 ng/mL) concentrations for MAA and AA; and low (10 ng/mL), medium (500 ng/mL), and high (4000 ng/mL) concentrations for tramadol and its metabolites. For tramadol and its metabolites, the calibration range was 1–5000 ng/mL, and for MAA and AA it was 800–40,000 ng/mL. These samples were used to determine absolute recovery and intra- and inter-day precision. Selectivity was evaluated by preparing the lower limit of quantification (LLOQ), set at 0.076 $\mu\text{g/mL}$ for MAA and AA and 0.8 ng/mL for tramadol and its metabolites in drug-free plasma. Stability was assessed for biological matrix stored at -70°C , at room temperature (20°C), and in samples processed in the autosampler. M2 and M5, due to their similar molecular weights to M1, were quantified as M1 equivalents.

1.6 Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated applying a non-compartmental model with PKSolver software (Zhang, Huo, Zhou, and Xie, 2010). The observed variables were as follows: terminal phase rate constant (λ_z), half-life ($T_{1/2}$), maximum plasma concentration (C_{max}), plasma concentration at time zero (C_0), time to reach C_{max} (T_{max}), area under the plasma concentration curve from zero to the time of the last measurable concentration ($\text{AUC}_{0 \rightarrow t}$) and the extrapolation of AUC to infinity ($\text{AUC}_{0 \rightarrow \infty}$), the ratio between $\text{AUC}_{0 \rightarrow t}$ to $\text{AUC}_{0 \rightarrow \infty}$, the mean residual time from moment zero to infinity ($\text{MRT}_{0 \rightarrow \infty}$), volume of distribution (V_d) which is the estimated volume of distribution based on the AUC, volume of distribution at steady state (V_{ss}) and the clearance (Cl).

1.7 Statistical Analysis

Statistical power was assessed using effect size estimation (Cohen's *d*) for key pharmacokinetic variables (C_{max} , $AUC_{0-\infty}$, and Cl_{obs}) to evaluate the sensitivity of the experimental design. Results indicated that, although the sample size per group ($n=9$) did not provide ideal power for all variables, it was sufficient to detect significant differences ($>80\%$ for $AUC_{0-\infty}$). Normality was evaluated for the specified parameters. Normally distributed data were analyzed using the *t*-test, while non-normal data were assessed using the Mann–Whitney test, with $p < 0.05$ considered statistically significant. Pearson correlation was performed for all parameters with significant differences. Linear Pearson correlation was applied to parameters showing statistical differences for AA. Statistical analyses were conducted using Python®.

Results

The method was revalidated to ensure data accuracy according to previous studies (de Macêdo et al., 2021; Arcoverde et al., 2023; Araújo-Silva et al., 2024). All calibration curves presented R^2 greater than 0.99, demonstrating linearity between equipment responses and curve concentrations. The method also exhibited satisfactory selectivity, reproducibility, and repeatability, with relative standard deviations below 5%. The samples were stable under the conditions used for analysis.

After intravenous administration of metamizole and metamizole-tramadol, metabolites were monitored for 48 hours in all animals (Figures 1, 2, 3 and 4). In G1, MAA was detectable and quantifiable for 48 hours in all animals; AA was detectable and quantifiable for 48 hours in seven animals. In G2, MAA was quantifiable for 48 hours in three animals and

detectable for 48 hours in seven animals, whereas AA was quantifiable for 24 hours in six animals and detectable for 48 hours in all animals. Tramadol was detectable and quantifiable for 24 hours in all animals; M1 was detectable for 48 hours in four animals and quantifiable for 12 hours in two animals; M2 was detectable for 36 hours in six animals and quantifiable for 10 hours in one animal; M5 was detectable for 10 hours in all animals and quantifiable for 4 hours in one animal.

Regarding the pharmacokinetic parameters analyzed, the data are presented in the tables below. Table 1 shows the results for the metabolite MAA, which exhibited statistically significant differences in C_{max} and C_0 .

Table 1. Pharmacokinetic parameters of 4-methylaminoantipyrine (MAA) following intravenous administration of $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone (G1) and $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone combined with $2 \text{ mg} \cdot \text{kg}^{-1}$ tramadol (G2) in dogs ($n = 9$). Values are presented as mean $\pm$ standard deviation and median (minimum–maximum).

Parameters	Unit	MAA	G1	G2	
			Metamizole	Metamizole 25 mg.kg ⁻¹ +	
			25 mg.kg ⁻¹	Tramadol 2 mg.kg ⁻¹	
		Mean ± SD	Median (mín-max)	Mean ± SD	Median (mín-max)
Lambda_z (λ_z)	1/h	0,07 ± 0,09	0,04 (0,02 – 0,29)	0,05 ± 0,05	0,03 (0,02 – 0,18)
T ½	H	19,53 ± 13,18	15,44 (2,35 – 38,58)	24,06 ± 12,85	25,57 (3,73 – 38,50)
Cmáx	µg/ml	233,52 ± 161,32*	171,72 (71,78 – 548,74)	59,62 ± 24,92*	60,95 (30,08 – 115,67)
C0	µg/ml	481,24 ± 558,14*	303,60 (77,45 – 1912,98)	73,00 ± 36,43*	66,60 (35,84 – 160,40)
Tmáx	H	-		-	
AUC_{0→t}	µg/ml*h	216,21 ± 114,91	183,75 (67,37 – 404,65)	179,31 ± 51,60	160,94 (116,25 – 259,05)
AUC_{0→∞}	µg/ml*h	240,60 ± 135,29	202,76 (70,00 – 441,35)	206,41 ± 63,49	185,10 (119,92 – 293,63)
AUC_{0→t/0→∞}		0,92 ± 0,07	0,91 (0,76 – 0,99)	0,88 ± 0,06	0,88 (0,79 – 0,96)
MRT_{0→∞}	H	12,25 ± 9,36	9,48 (2,02 – 32,43)	18,21 ± 7,85	18,68 (4,47 – 26,24)
Vd	(L)	208,54 ± 122,76	194,84 (70,91 – 908,30)	276,84 ± 143,00	305,85 (74,95 – 482,60)
Cl	(L/h)	10,22 ± 7,53	8,22 (3,73 – 23,81)	8,78 ± 2,69	9,00 (5,67 – 13,89)

Legend: Lambda_z: terminal phase rate constant; T1/2: half-life; CMax: maximum plasma concentration; C0: plasma concentration at time zero; TMax: time to reach maximum plasma concentration; AUC_{0→t}: area under the curve from time zero to the last measurable concentration; AUC_{0→∞}: area under the curve from time zero to

infinity; $AUC_{0 \rightarrow t} / AUC_{0 \rightarrow \infty}$: ratio of $AUC_{0 \rightarrow t}$ to $AUC_{0 \rightarrow \infty}$; $MRT_{0 \rightarrow \infty}$: mean residence time from time zero to infinity; V_d : apparent volume of distribution; Cl : total body clearance.

*Indicates a significant difference.

Table 2 presents the results for the metabolite AA. Statistically significant differences between G1 and G2 were observed for $AUC_{0 \rightarrow t}$, $AUC_{0 \rightarrow \infty}$, $MRT_{0 \rightarrow \infty}$ and Cl .

Table 2. Pharmacokinetic parameters of 4-aminoantipyrine (AA) following intravenous administration of $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone (G1) and $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone combined with $2 \text{ mg} \cdot \text{kg}^{-1}$ tramadol (G2) in dogs ($n = 9$). Values are presented as mean $\pm$ standard deviation and median (minimum–maximum).

AA	Unit	G1	G2		
		Metamizole 25 mg.kg ⁻¹	Metamizole 25 mg.kg ⁻¹ + Tramadol 2 mg.kg ⁻¹		
Parameters	Unit	Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)
Lambda_z (λz)	1/h	0,11 ± 0,03	0,11 (0,08 – 0,15)	0,11 ± 0,03	0,10 (0,07 – 0,17)
T ½	H	6,43 ± 1,63	6,23 (4,37 – 8,25)	6,82 ± 1,71	6,81 (4,04 – 9,02)
Cmáx	µg/ml	2,75 ± 1,52	2,56 (1,10 – 5,50)	3,48 ± 0,65	3,64 (2,46 – 4,41)
C0	µg/ml	-			
Tmáx	H	6,44 ± 2,40	6,00 (4,00 – 10)	6,61 ± 3,41	6,00 (1,5 – 12)
AUC_{0→t}	µg/ml*h	42,52 ± 20,61*	41,92 (12,77 – 79,02)	67,71 ± 20,63*	61,30 (44,03 – 109,36)
AUC_{0→∞}	µg/ml*h	43,23 ± 20,56*	42,66 (13,37 – 79,65)	69,01 ± 21,51*	62,20 (45,40 – 113,17)
AUC_{0→t} / 0→∞		0,98 ± 0,02	0,98 (0,95 – 0,99)	0,98 ± 0,01	0,98 (0,96 – 0,99)
MRT_{0→∞}	H	11,79 ± 2,87*	13,15 (6,90 – 14,83)	14,50 ± 2,20*	15,13 (10,78 – 18,02)
Vd	(L)	460,85 ± 272,43	335,07 (187,91 – 843,82)	255,72 ± 105,89	221,65 (166,70 – 477,85)
Cl	(L/h)	50,67 ± 32,72*	39,07 (20,92 – 124,60)	26,07 ± 7,20*	26,79 (14,72 – 36,71)

Legend: Lambda_z: terminal phase rate constant; T1/2: half-life; CMax: maximum plasma concentration; C0: plasma concentration at time zero; TMax: time to reach maximum plasma concentration; AUC_{0→t}: area under the curve from time zero to the last measurable concentration; AUC_{0→∞}: area under the curve from time zero to infinity; AUC_{0→t}/AUC_{0→∞}: ratio of AUC_{0→t} to AUC_{0→∞}; MRT_{0→∞}: mean residence time from time zero to infinity; Vd: apparent volume of distribution; Cl: total body clearance.

*Indicates a significant difference.

The pharmacokinetic parameters of AA that showed statistical differences were evaluated for linear correlation. A Pearson linear correlation was observed between AUC and Cl, showing a strong negative correlation ($R^2 = -0.81$; $p < 0.001$; $Cl = -7.7 \times 10^{-10} \times AUC_{0-t} + 2.93 \times 10^{-4}$).

A violin plot (Figure 5) was generated to represent the AA metabolite variables that exhibited statistically significant differences.

Table 3 presents the descriptive pharmacokinetic parameters of tramadol and a comparison with selected studies reported in the literature.

Table 3. Pharmacokinetic parameters of tramadol following intravenous administration of 2 mg·kg⁻¹ tramadol combined with 25 mg·kg⁻¹ dipyrone (n = 9) in dogs. Values are presented as mean ± standard deviation and median (minimum–maximum). Comparison with previously published studies (Kukanich, Papich, 2004; McMillan *et al.*, 2008; Vettorato *et al.*, 2010; Giorgi *et al.*, 2010) (Dose-dependent values were adjusted to 2 mg·kg⁻¹ for comparison).

G2	Metamizole 25 mg.kg ⁻¹ + Tramadol 2 mg.kg ⁻¹			Kukanich; Papich, 2004 4,4 mg.kg ⁻¹	McMillan et al., 2008 2 mg.kg ⁻¹	Vettorato et al., 2010 2 mg.kg ⁻¹	Giorgi et al., 2010 4 mg.kg ⁻¹
Parameters	Unit	Mean ± SD	Median (min - max)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Lambda_z (λz)	1/h	0,11 ± 0,08	0,09 (0,02 – 0,28)	0,89 ± 0,13	-	-	-
T ½	H	10,30 ± 8,30	8,05 (2,42 – 31,13)	0,80 ± 0,12	1,04 ± 0,42	2,24 ± 0,87	0,41 ± 0,39
Cmáx	µg/ml	1,11 ± 0,42	1,07 (0,57 – 1,97)	-	-	-	-
C0	µg/ml	0,32 ± 0,69	0,010 (0,004 – 2,00)	0,77 ± 0,18	-	5,48 ± 5,92	-
Tmáx	H	-	-	-	-	-	-
AUC0→t	µg/ml*h	2,17 ± 0,96	1,90 (1,11 – 3,92)		0,82 ± 0,29	-	-
AUC 0→∞	µg/ml*h	2,25 ± 0,95	2,00 (1,14 – 3,96)	0,13 ± 0,02	-	3,56 ± 0,67	2,16 ± 0,79
AUC0→t/0→∞		0,96 ± 0,04	0,98 (0,84 – 0,99)	-	-	-	-
MRT0→∞	H	4,97 ± 3,16	3,98 (2,65 – 13,53)	0,93 ± 0,12	-	2,63 ± 1,13	1,13 ± 1,13
Vd	(L)	1027,89 ± 810,67	977,16 (217,08 – 2982,10)	0,3 ± 0,04	0,05 ± 0,01	9,56 ± 5,17	0,11 ± 0,03
Cl	(L/h)	69,89 ± 29,74	66,40 (33,63 – 116,46)	49,17 ± 7,37	40,61 ± 14,03	26,23 ± 18,60	13,85 ± 6,90

Legend: Lambda_z: terminal phase rate constant; T1/2: half-life; CMax: maximum plasma concentration; C0: plasma concentration at time zero; TMax: time to reach maximum plasma concentration; AUC0→t: area under the curve from time zero to the last measurable concentration; AUC0→∞: area under the curve from time zero to

infinity; $AUC_{0 \rightarrow t} / AUC_{0 \rightarrow \infty}$: ratio of $AUC_{0 \rightarrow t}$ to $AUC_{0 \rightarrow \infty}$; $MRT_{0 \rightarrow \infty}$: mean residence time from time zero to infinity; V_d : apparent volume of distribution; Cl : total body clearance.

Table 4 presents the descriptive pharmacokinetic parameters of the M1 metabolite and a comparison with selected studies reported in the literature.

Table 4. Pharmacokinetic parameters of O-desmethyltramadol (M1) following intravenous administration of $2 \text{ mg} \cdot \text{kg}^{-1}$ tramadol combined with $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone ($n = 9$) in dogs. Values are presented as mean $\pm$ standard deviation and median (minimum–maximum). Comparison with previously published studies (Vettorato *et al.*, 2010; Giorgi *et al.*, 2010). (Dose-dependent values were adjusted to $2 \text{ mg} \cdot \text{kg}^{-1}$ for comparison).

Parameters	Unit	M1		Vetoratto et al., 2010 2 mg.kg ⁻¹	Giorgi et al., 2010 4 mg.kg ⁻¹
		Mean ± SD	Median (min - max)	Mean ± SD	Mean ± SD
Lambda_z (λz)	1/h	0,09 ± 0,05	0,06 (0,02 – 0,15)	3,59 ± 0,88	0,52
T ½	h	11,55 ± 8,07	11,01 (4,52 – 28,66)		1,34
Cmáx	µg/ml	0,012 ± 0,006	0,012 (0,003 – 0,02)	0,35 ± 0,17	0,011 ± 0,002
C0	µg/ml	-			
Tmáx	h	0,90 ± 0,56	0,75 (0,25 – 1,5)	0,29 ± 0,16	1,70 ± 1,34
AUC0→t	µg/ml*h	0,06 ± 0,03	0,05 (0,03 – 0,14)		
AUC 0→∞	µg/ml*h	0,06 ± 0,03	0,05 (0,03 – 0,16)	1,24 ± 0,16	0,013
AUC0→t/0→∞		0,92 ± 0,08	0,94 (0,77 – 0,99)		
MRT0→∞	h	11,46 ± 5,59	9,80 (4,48 – 21,84)	5,42 ± 2,54	2,18
Vd	(L)	35147,95 ± 20156,35	27287,94 (16152,93 - 67157,48)	-	-
Cl	(L/h)	2411,58 ± 909,93	2551,15 (825,46 - 3762,00)	-	-

Legend: Lambda_z: terminal phase rate constant; T1/2: half-life; CMax: maximum plasma concentration; C0: plasma concentration at time zero; TMax: time to reach maximum plasma concentration; AUC0→t: area under the curve from time zero to the last measurable concentration; AUC0→∞: area under the curve from time zero to

infinity; $AUC_{0 \rightarrow t} / AUC_{0 \rightarrow \infty}$: ratio of $AUC_{0 \rightarrow t}$ to $AUC_{0 \rightarrow \infty}$; $MRT_{0 \rightarrow \infty}$: mean residence time from time zero to infinity; V_d : apparent volume of distribution; Cl : total body clearance.

Table 5 presents the descriptive pharmacokinetic parameters of the M2 and M5 metabolites and a comparison with selected studies reported in the literature.

Table 5. Pharmacokinetic parameters of N-desmethyltramadol (M2) and N,O-desmethyltramadol (M5) following intravenous administration of $2 \text{ mg} \cdot \text{kg}^{-1}$ tramadol combined with $25 \text{ mg} \cdot \text{kg}^{-1}$ dipyrone ($n = 9$) in dogs. Values are presented as mean $\pm$ standard deviation and median (minimum–maximum) (Giorgi *et al.*, 2010).

G2 Tramadol	Metamizole 25 mg.kg ⁻¹ + Tramadol 2 mg.kg ⁻¹	M2		Giorgi et al., 2010 4 mg.kg ⁻¹	M5		Giorgi et al., 2010 4 mg.kg ⁻¹
Parameters	Unit	Mean ± SD	Median (min - max)	Mean ± SD	Mean ± SD	Median (min - max)	Mean ± SD
Lambda_z (λz)	l/h	0,12 ± 0,11	0,07 (0,04 – 0,38)	0,27 ± 0,07	0,14 ± 0,07	0,12 (0,08 – 0,28)	0,26 ± 0,07
T ½	H	8,78 ± 4,83	9,46 (1,80 – 15,83)	2,63 ± 0,55	5,56 ± 1,89	5,95 (2,41 – 7,79)	2,79 ± 0,70
Cmáx	µg/ml	0,006 ± 0,003	0,006 (0,002 – 0,01)	0,17 ± 0,05	0,001 ± 0,001	0,001 (0,0006 – 0,007)	0,19 ± 0,02
C0	µg/ml	-			-		
Tmáx	H	1,40 ± 0,58	1,50 (0,25 – 1,5)	0,0004 ± 0,0002	1,33 ± 0,43	1,50 (0,50 – 2,00)	0,0004 ± 0,0003
AUC0→t	µg/ml*h	0,03 ± 0,01	0,03 (0,01 – 0,05)	-	0,006 ± 0,003	0,004 (0,003 – 0,01)	
AUC 0→∞	µg/ml*h	0,03 ± 0,01	0,03 (0,01 – 0,05)	0,59 ± 0,21	0,006 ± 0,004	0,004 (0,003 – 0,014)	0,78 ± 0,15
AUC0→t/0→∞		0,98 ± 0,01	0,99 (0,96 – 0,99)		0,96 ± 0,02	0,96 (0,93 – 0,99)	
MRT0→∞	H	5,75 ± 1,55	5,83 (3,12 – 8,88)	3,83 ± 0,76	5,36 ± 1,54	6,09 (2,84 – 7,35)	4,32 ± 0,72
Vd	(L)	52058,27 ± 30217,92	39051,15 (22223,26 - 98895,48)	-	209180,65 ± 102742,84	184715,36 (48255,32 - 359925,50)	-
Cl	(L/h)	10154,60 ± 9452,40	4655,25 (2428,75 - 49923,63)	-	25500,51 ± 11313,00	27046,58 (9120,14 - 43542,08)	-

Legend: λ_z : terminal phase rate constant; $T_{1/2}$: half-life; C_{Max} : maximum plasma concentration; C_0 : plasma concentration at time zero; T_{Max} : time to reach maximum plasma concentration; $AUC_{0 \rightarrow t}$: area under the curve from time zero to the last measurable concentration; $AUC_{0 \rightarrow \infty}$: area under the curve from time zero to infinity; $AUC_{0 \rightarrow t}/AUC_{0 \rightarrow \infty}$: ratio of $AUC_{0 \rightarrow t}$ to $AUC_{0 \rightarrow \infty}$; $MRT_{0 \rightarrow \infty}$: mean residence time from time zero to infinity; V_d : apparent volume of distribution; Cl : total body clearance.

Regarding behavioral and adverse effects, in G1, three animals exhibited tachypnea, while in G2, three animals showed nausea and one exhibited hypersalivation, occurring shortly after the start of drug administration, with a mean duration of 15 minutes in both groups.

Discussion

In routine clinical management of pain in dogs in Brazil, the combination of dipyrone and tramadol is still common. However, no studies have evaluated the pharmacokinetic profile of this drug combination in dogs, making this a pioneering study relevant for understanding potential interactions between these drugs. It was possible to quantify the metabolites of both compounds.

Regarding available pharmacokinetic studies in the literature, López-Muñoz et al. (2013) investigated different combinations of dipyrone and tramadol doses in rats with arthritis, using single administrations and evaluating antinociceptive effects and dose–response curves. Results showed that the combination produced additive or potentiating effects, and the authors suggested a possible pharmacokinetic interaction between the drugs, although the nature of this interaction was not detailed. They proposed that the drugs could compete for the same

cytochrome P450 enzymes (glucuronidation pathway), leading to alterations in metabolite concentrations and pharmacological effects, suggesting further studies.

In this context, Moreno-Rocha et al. (2015) used one of the previously tested dose combinations to analyze the pharmacokinetics and pharmacodynamics of dipyrone alone and in combination with tramadol in rats with induced arthritis. The authors found no significant differences in pharmacokinetic parameters for MAA or AA following a single administration between treatments, indicating that tramadol did not alter the pharmacokinetics of metamizole metabolites when co-administered in a single dose. Significant differences were observed only after multiple daily doses (4 consecutive days), suggesting the need for additional studies with multiple-dose regimens in other species.

Although Moreno-Rocha et al. (2015) reported no significant pharmacokinetic differences after a single administration, a trend toward lower MAA C_{max} was observed when dipyrone was combined with tramadol. In the present study, this reduction was significant, with C_{max} and C₀ markedly lower in the combination group. These findings indicate a pharmacokinetic interaction occurring at an early stage of drug disposition.

Considering the intravenous route and the rapid conversion of dipyrone to MAA, the reduction in peak concentrations is more consistent with alterations in the initial appearance of MAA in the systemic circulation rather than changes in overall exposure, as AUC remained unchanged. This profile suggests a redistribution of exposure over time, which may contribute to a lower likelihood of acute concentration-dependent adverse effects without compromising sustained MAA plasma levels.

Araújo-Silva et al. (2024) conducted a pharmacokinetic study of dipyrone alone and in combination with tramadol in donkeys using similar doses to the present study. For MAA, results indicated a (non-significant) increase in T_{1/2} when the drugs were combined. In

the present study, although the increase was not significant, $T_{1/2}$ also increased with the combination. It is important to note that this study continues from a previous publication (Mouta *et al.*, 2025), which identified differences between ultrarapid metabolizers and normal/rapid metabolizers. The variation in dipyrene metabolism between rapid and slow metabolizers influenced tramadol metabolism, leading to the observed variations in $T_{1/2}$, C_{max} , AUC and Cl. The mean values in the present study were described based on all animals, without distinguishing metabolizer groups.

Regarding MAA, a slight reduction in AUC was observed in G2, without significant differences, suggesting that the combination did not alter dipyrene bioavailability. The minimum plasma concentrations of MAA required to inhibit 50% of cyclooxygenase activity (IC_{50} – COX-1 and COX-2) have been reported in humans, with values of 0.553 $\mu\text{g/mL}$ for IC_{50} -COX1 and 0.926 $\mu\text{g/mL}$ for IC_{50} -COX2 (Hinz *et al.*, 2007; Knights *et al.*, 2010). In G1, three animals maintained plasma concentrations above these values for 48 hours, whereas in G2, six animals exceeded these thresholds. These results indicate that dipyrene alone or in combination with tramadol achieved the minimum inhibitory concentrations of COX-1 and COX-2 for MAA.

Regarding the AA metabolite, $AUC_{0 \rightarrow t}$ and $AUC_{0 \rightarrow \infty}$ increased significantly in G2, suggesting enhanced AA bioavailability. A significant reduction in Cl was also observed. The decreased Cl resulted in increased bioavailability, possibly due to impaired AA elimination. Competition between cytochrome P450 (CYP) enzymes may have reduced AA clearance. This pharmacokinetic interaction should be evaluated in future studies using multiple-dose regimens, as it may be relevant during prolonged treatment and could differ between single- and multiple-dose administrations (Moreno-Rocha *et al.*, 2015). $MRT_{0 \rightarrow \infty}$ of AA also increased significantly in G2, suggesting that the metabolite remained longer in circulation due to the reduced Cl.

In the previously mentioned rat study, no animals exhibited adverse effects resulting from the drug combination. In donkeys, adverse effects such as restlessness and ataxia were observed in two animals (Araújo-Silva et al., 2024) and in the present study, some animals showed nausea and hypersalivation with the drug combination. These signs have been previously reported in other studies using tramadol in different species, including donkeys (Mouta et al., 2021) and horses (Knych *et al.*, 2011).

Although a group receiving tramadol alone was not included, the pharmacokinetic profile of this agent and its main metabolites when combined with dipyrone was described in this study and compared with previous reports (Tables 3–5). According to Domínguez-Oliva et al. (2021), dogs produce higher concentrations of M2 than M1. However, in the present study, M1 concentrations were higher than those of M2 and M5, which differs from previously reported intravenous tramadol studies in dogs (Giorgi et al., 2010). This difference may be explained by enzymatic competition promoted by the combination, specifically along the N-demethylation pathway, resulting in reduced formation of M2 and M5. MAA is converted to AA via CYP3A4 and CYP2B6, which may reduce M2 formation and favor O-demethylation, thereby also limiting M5 production. These findings are supported by the lower observed M5 concentrations. Dogs produce M2 primarily through CYP2B11 (Perez-Jimenez et al., 2016), which may be homologous to the CYP enzymes involved in metamizole metabolism.

Indirect quantification of metabolites using LC-MS/MS in the absence of pure standards is feasible but requires methodological caution and validation (Ghafari; Sleno, 2024). The use of calibration curves from structurally related compounds can provide conceptual support, although analytical responses may vary among metabolites; therefore, results should be interpreted as approximate estimates (Kruve, 2019). In the specific case of tramadol, although there is no direct evidence for using the M1 calibration curve to quantify M2 and M5, the literature suggests that this approach is viable if the method is validated, including

assessment of accuracy and precision. Thus, the use of the M1 calibration curve as a substitute for M2 and M5 should be accompanied by validation experiments to confirm applicability and minimize errors arising from differences in ionization responses and matrix effects.

Regarding tramadol, compared to previous studies, reductions in C₀ and increases in MRT and T_{1/2} were observed. These findings may indicate a distributive interaction. The reduction in C₀ suggests that the combined drug was distributed more rapidly into tissues, lowering initial plasma concentration. Increases in MRT and T_{1/2} may indicate that drug redistributed from tissues back into circulation slowly. A metabolic interaction at the binding site may also have occurred, with the increase suggesting that dipyrone inhibited tramadol metabolism, prolonging its presence in the organism.

For M1, an increase in T_{1/2} and a reduction in drug bioavailability were observed when administered with dipyrone. This suggests that combining dipyrone with tramadol may not be advantageous if the goal is to increase plasma concentrations of M1. Notably, tramadol concentrations increased substantially. Future studies evaluating tramadol's analgesic profile in dogs are necessary to determine whether analgesia derives primarily from M1 (the active metabolite) as in other species, or from the parent drug.

The minimum effective concentration (MEC) for analgesia of tramadol and M1 has not yet been described in dogs, however, in humans, following surgery, MEC of tramadol were found to range from 298 (127-469) ng/mL (Lehmann et al., 1990) to 590 (180-1000) ng/mL (Grond et al., 1999), and MEC of M1 were found to range from 39.6 (10.1-69.1) ng/mL (Lehmann et al., 1990) to 84 (50-134) ng/mL (Grond et al., 1999). If these concentrations apply to dogs, in the present study, the C_{max} for tramadol was substantially higher than the MEC established for humans, suggesting that therapeutic levels were achieved. However, the C_{max} for M1 was remaining below the human therapeutic range. This indicates that the analgesic

effect in these animals may be primarily driven by the parent drug rather than the M1 metabolite, or that the species-specific MEC for M1 differs from that of humans. Of course species-specific analgesiometric testing is ultimately needed in order to assess tramadol and M1 efficacy in dogs.

Regarding the clinical efficacy of tramadol, current evidence and international guidelines support its role within multimodal analgesic strategies, despite its recognized pharmacokinetic variability in the canine species. The 2022 AAHA Pain Management Guidelines categorize tramadol as an adjunctive therapy for acute pain, emphasizing that it should be part of a tiered, multimodal approach rather than a standalone treatment (Gruen et al., 2022). Similarly, the 2022 WSAVA guidelines include tramadol in their analgesic protocols, particularly in scenarios where access to full μ -opioid agonists is restricted, reinforcing its importance in global veterinary practice (Monteiro et al., 2022). Furthermore, a systematic review and meta-analysis by Donati et al. (2021) demonstrated that tramadol administration likely reduces the need for rescue analgesia compared to placebo in postoperative settings. While the metabolic profile described in the present study reflects the known individual variation in dogs, these international references justify the clinical use of tramadol as a viable component for managing acute pain in Brazil.

The doses used in the present study were based on clinical usage and previous studies. The same doses were applied by Flôr et al. (2013), who evaluated dipyrone/tramadol, tramadol alone, and dipyrone/carprofen combinations for chronic oncologic pain control over 14 days using pain scales and quality-of-life assessments. They reported that the dipyrone/tramadol combination was effective and safe, and that adding an anti-inflammatory agent did not confer additional benefit in most animals.

Teixeira et al. (2013) evaluated a combination of tramadol ($3 \text{ mg}\cdot\text{kg}^{-1}$) and dipyrone ($30 \text{ mg}\cdot\text{kg}^{-1}$) intravenously. Analgesic efficacy was assessed in the immediate postoperative period in bitches undergoing unilateral mastectomy with or without ovariohysterectomy. The combination provided effective analgesia for up to 24 hours, with pain scores comparable to groups treated with tramadol alone or tramadol combined with meloxicam. In the tramadol/dipyrone combination, pain scores remained above baseline for up to 8 hours, and the number of animals requiring rescue analgesia was slightly lower than in the tramadol-alone group, although no significant differences were observed. These clinical studies support the rationale for selecting these agents in the present research.

The limitations of this study include the absence of a tramadol-only control group for direct comparison and the indirect quantification of M2 and M5 metabolites, necessitated by the unavailability of analytical standards.

Conclusion

A significant pharmacokinetic interaction exists between metamizole and tramadol, reflected by reduced elimination and increased AA bioavailability. These findings suggest that the combination may alter drug exposure, potentially influencing the duration and intensity of analgesia. However, as the most severe adverse effects of metamizole are primarily attributed to MAA rather than AA, the direct clinical implications for safety should be interpreted with caution. Additionally, inhibition of tramadol conversion to M1 was observed.

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Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Author Contributions

A.N.M. : Data acquisition, preparation of the original work. K.N.A, R.A.H., N.S.F., Y.D.B.P., C.V.A.O. : Data acquisition. G.A-S, V.V.P: developed the applied methodology, performed the statistical analyses, supervised the work and reviewed the final manuscript.

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Figure legends

Figure 1: Log of the plasma concentration vs. time for 4-metilaminoantipirina (MAA) following intravenous administration of metamizole ($25 \text{ mg}\cdot\text{kg}^{-1}$) alone and in association with tramadol ($2 \text{ mg}\cdot\text{kg}^{-1}$) in nine healthy dogs.

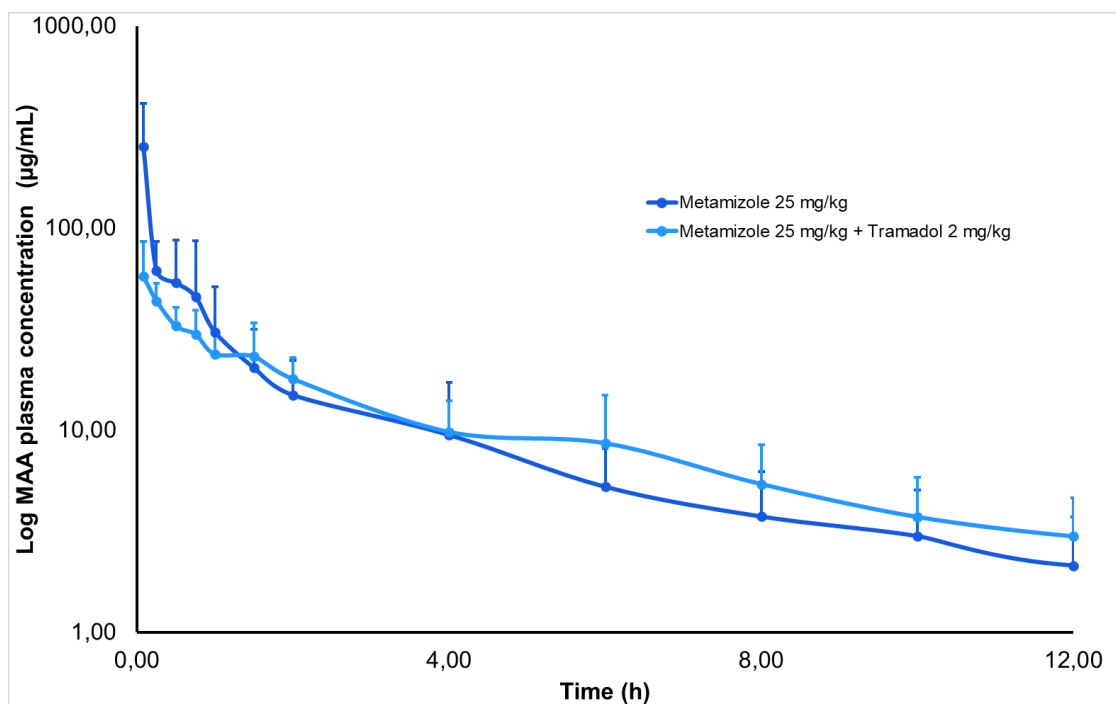


Figure 2: Plasma concentration vs. time for 4-aminoantipyrine (AA) following intravenous administration of metamizole (25 mg·kg⁻¹) alone and in association with tramadol (2 mg·kg⁻¹) in nine healthy dogs.

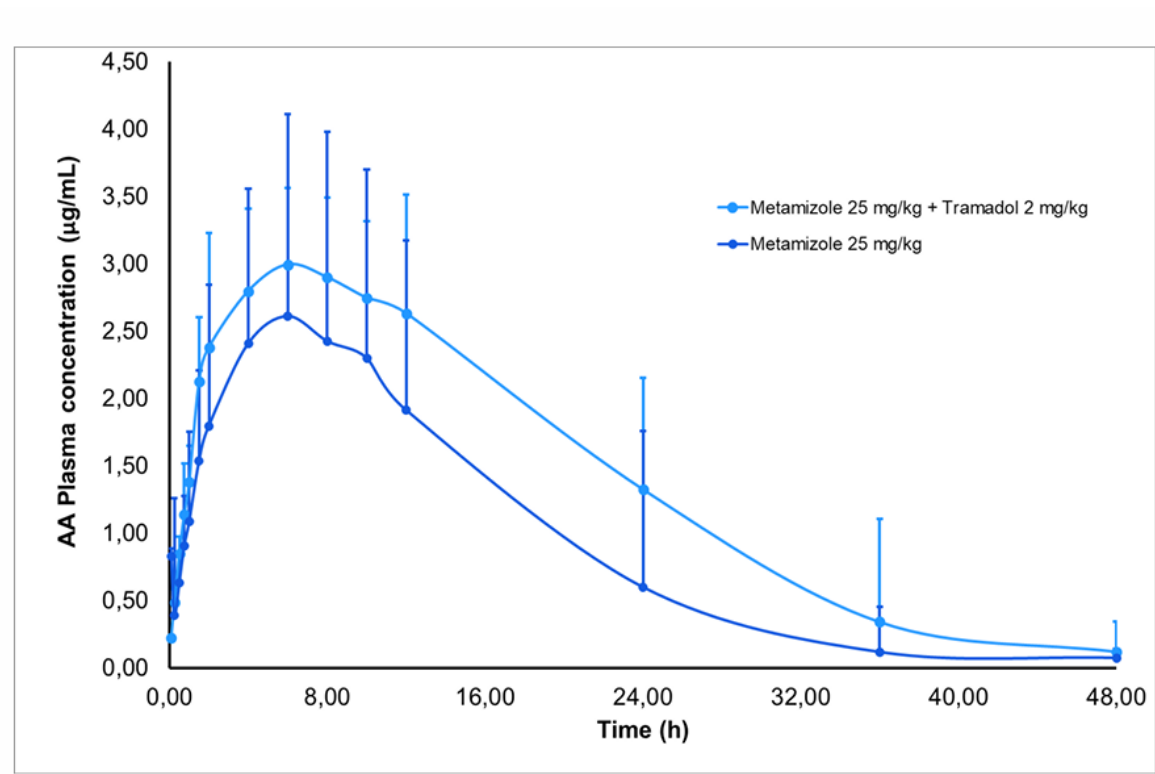


Figure 3. Plasma concentration vs. time for tramadol ($2 \text{ mg}\cdot\text{kg}^{-1}$) following intravenous administration with metamizole ($25 \text{ mg}\cdot\text{kg}^{-1}$) in nine healthy dogs.

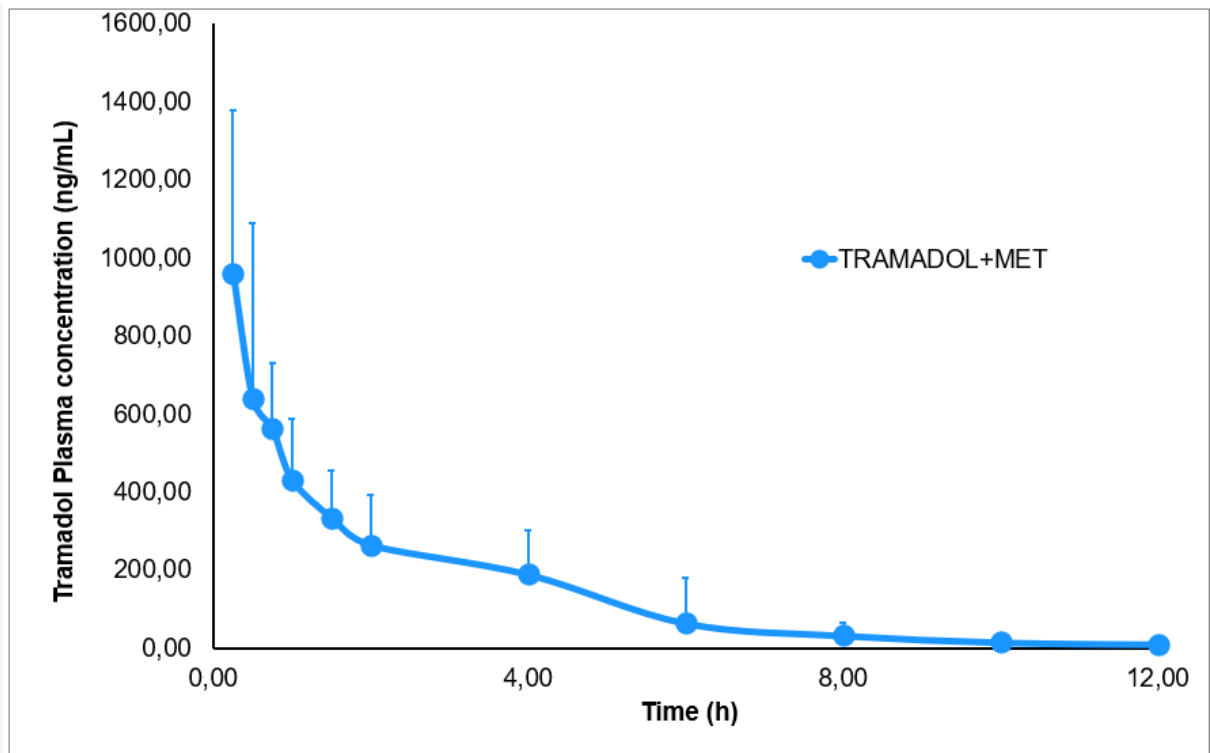


Figure 4. Plasma concentration vs. time for O-desmetiltramadol (M1) ($2\text{ mg}\cdot\text{kg}^{-1}$) following intravenous administration with metamizole ($25\text{ mg}\cdot\text{kg}^{-1}$) in nine healthy dogs.

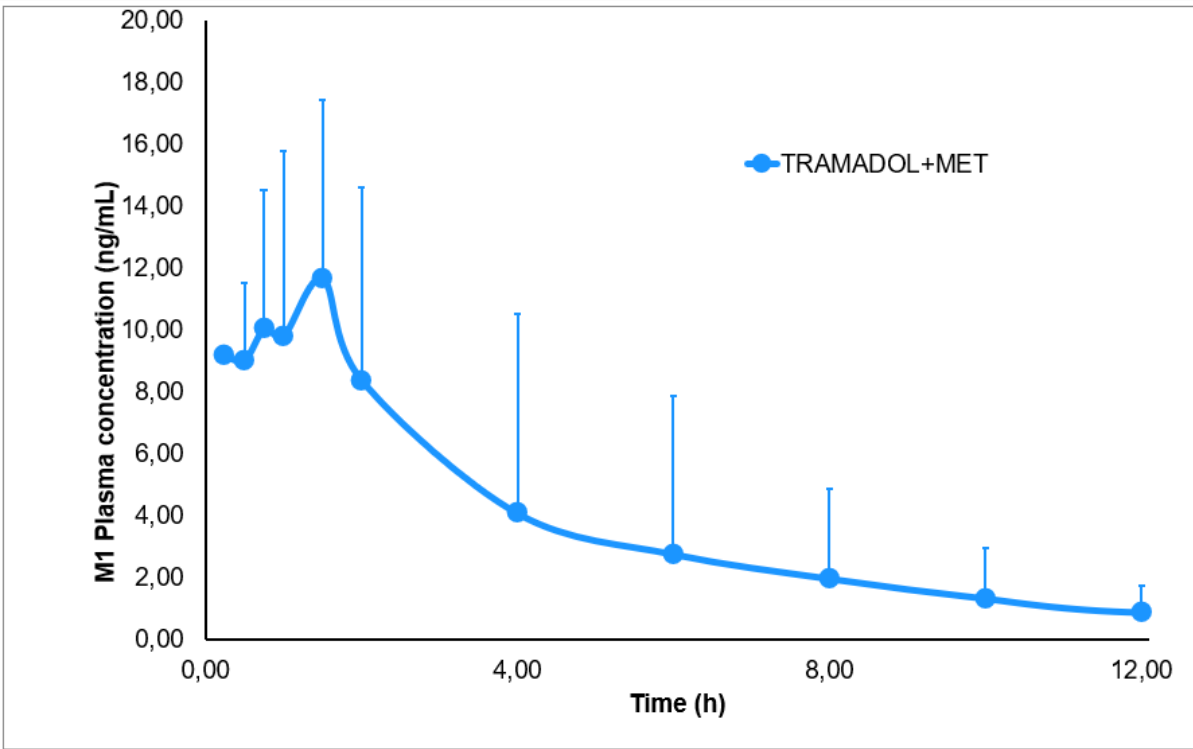
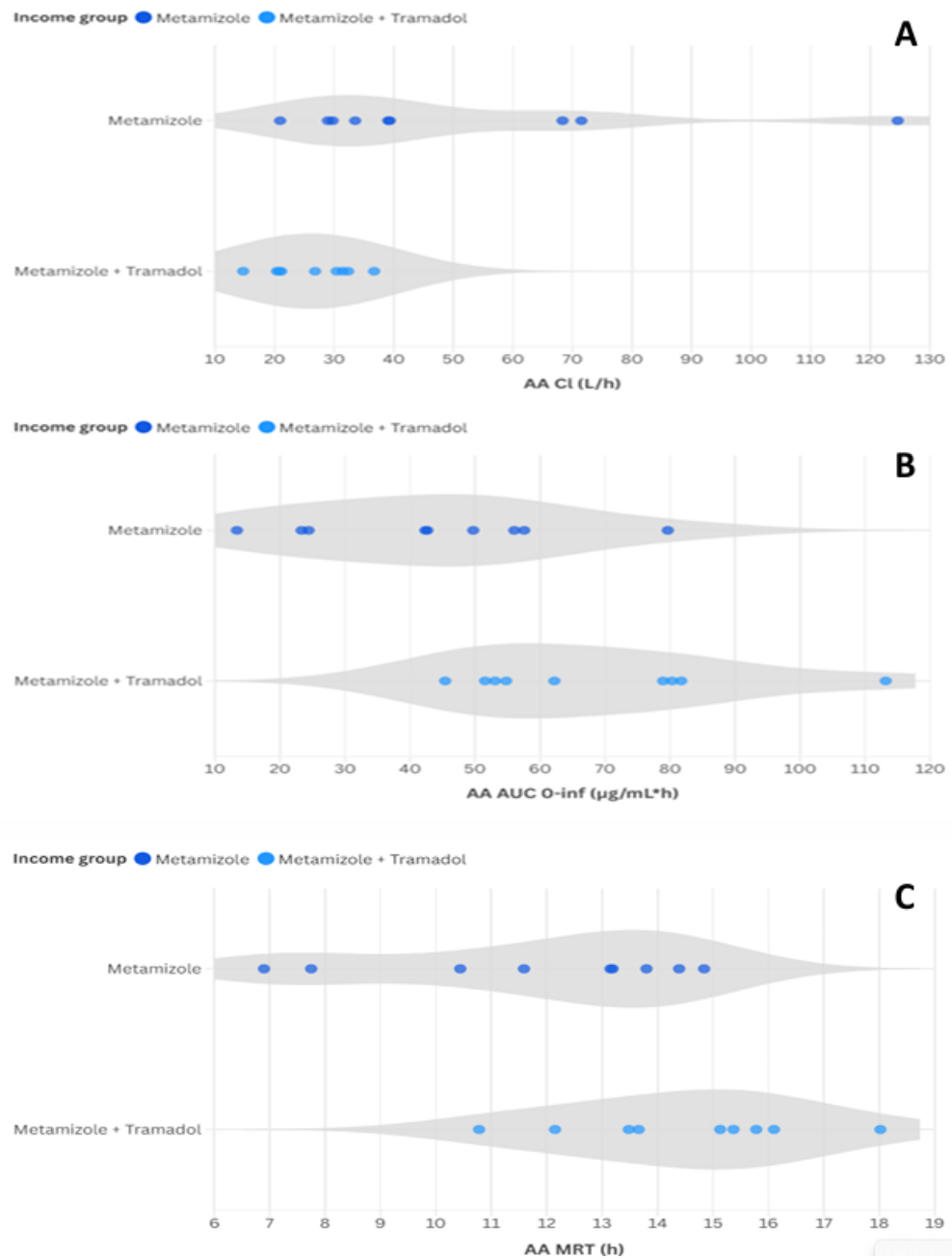


Figure 5. AA variables showing statistically significant differences between the metamizole group (G1) and the metamizole plus tramadol group (G2). A. Cl (L/h); B. AUC_{0→∞} (μg/mL·h); C. MRT_{0→∞} (h).



6. CONSIDERAÇÕES FINAIS

A presente tese cumpriu com os objetivos propostos, validando metodologias bionalíticas por UPLC-MS/MS que permitiram a quantificação prolongada dos metabólitos da dipirona. Os resultados demonstraram que a farmacocinética da dipirona é influenciada tanto por fatores intrínsecos do indivíduo quanto pela interação com outros fármacos. Os resultados revelaram, pela primeira vez, a existência de uma variabilidade fenotípica no metabolismo da dipirona em cães, permitindo a classificação dos animais em metabolizadores lentos (ML) e normais/rápidos (MN), preenchendo uma lacuna crítica na farmacocinética. A detecção dos metabólitos ativos (MAA e AA) por até 48 horas sugere que a dipirona pode manter concentrações plasmáticas capazes de inibir a cicloxigenase por períodos prolongados, especialmente no grupo de metabolizadores lentos, o que cabem discussões para a individualidade de protocolos na clínica veterinária.

Quanto à associação terapêutica, a coadministração com o tramadol evidenciou uma interação farmacocinética. A hipótese do aumento da meia vida com a associação dos fármacos foi confirmada. Embora o tramadol tenha aumentado a biodisponibilidade do metabólito AA, a dipirona atuou interferindo na formação do metabólito M1. Embora clinicamente comum, a associação altera o perfil farmacocinético de ambos os fármacos em relação às suas administrações isoladas. Os efeitos adversos foram observados em apenas três animais, e sem alta relevância clínica.

Esta pesquisa estabelece que o uso da dipirona em cães deve considerar a individualidade metabólica e o potencial de interações medicamentosas. Tais evidências indicam que a simples extrapolação de doses pode não ser ideal e reforça a necessidade de estudos farmacogenéticos em cães.

ANEXOS



UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Mossoró, 02 de maio de 2023.

PARECER 07/2023

Certificamos que o projeto intitulado “Perfil farmacocinético dos metabólitos ativos da dipirona, 4-metilaminoantipirina (MAA) e 4-aminoantipirina (AA), isolados e associados ao tramadol em cães” sob a responsabilidade de VALÉRIA VERAS DE PAULA, o qual envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da lei 11.794 de 8 de outubro de 2009 e com as normas editadas pelo Conselho Nacional de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFERSA em reunião no dia 14 de abril de 2023.

<i>Vigência do projeto</i>	<i>Maio a agosto / 2023</i>
<i>Espécie/linhagem</i>	<i>Canina / SRD</i>
<i>N. de Animais</i>	<i>40</i>
<i>Peso/idade</i>	<i>15 a 25 kg / 1 ano</i>
<i>Sexo</i>	<i>machos</i>
<i>Origem</i>	<i>Hospital Veterinário da UFERSA</i>

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Coordenador CEUA-UFERSA