Fundação Universidade Federal de Mato Grosso do Sul
Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição
Programa de Pós-Graduação em Ciências Farmacêuticas

PATRÍCIA ESPINOSA DOS SANTOS

DEVELOPMENT AND VALIDATION OF A METHOD FOR THE SIMULTANEOUS DETERMINATION OF SULFAMETHAZINE, TRIMETHOPIM AND DOXYCYCLINE IN VETERINARY FORMULATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Desenvolvimento e Validação de Método para Determinação Simultânea de Sulfametazina, Trimetoprima e Doxiciclina em Formulação Veterinária utilizando Cromatografia Líquida de Alta Eficiência

CAMPO GRANDE - MS
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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal de Mato Grosso do Sul, como requisito para obtenção do título de Doutor em Ciências Farmacêuticas, área de concentração: controle de qualidade.

Orientadora: Profa. Dra. Nájla Mohamad Kassab.

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Prof. Dr. Teófilo Fernando Mazon Cardoso - UFMS (FACFAN)

dos Santos, Patrícia Espinosa

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ATA DE DEFESA DE TESE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DOUTORADO

Ao primeiro dia do mês de dezembro do ano de dois mil e vinte e três, às oito horas, via Google Meet, na Fundação Universidade Federal de Mato Grosso do Sul, reuniu-se a Banca Examinadora composta pelos membros: Najla Mohamad Kassab (UFMS), Andreia Peraro do Nascimento (UFJF), Everton do Nascimento Alencar (UFMS), Joao Batista Gomes de Souza (UFMS) e Teofilo Fernando Mazon Cardoso (UFMS), sob a presidência do primeiro, para julgar o trabalho da aluna: PATRÍCIA ESPINOSA DOS SANTOS, CPF 05079006188, do Programa de Pós-Graduação em Ciências Farmacêuticas, Curso de Doutorado, da Fundação Universidade Federal de Mato Grosso do Sul, apresentado sob o título "DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO PARA DETERMINAÇÃO SIMULTÂNEA DE SULFAMETAZINA, TRIMETOPRIMA E DOXICICLINA EM FORMULAÇÃO VETERINÁRIA UTILIZANDO CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA" e orientação de Najla Mohamad Kassab. A presidente da Banca Examinadora declarou abertos os trabalhos e agradeceu a presença de todos os Membros. A seguir, concedeu a palavra à aluna que expôs sua Tese. Terminada a exposição, os senhores membros da Banca Examinadora iniciaram as arguições. Terminadas as arguições, a presidente da Banca Examinadora fez suas considerações. A seguir, a Banca Examinadora reuniu-se para avaliação, e após, emitiu parecer expresso conforme segue:

EXAMINADOR

Dra. NAJLA MOHAMAD KASSAB (Interno)
Dra. ANDREIA PERARO DO NASCIMENTO (Externo)
Dr. JOAO BATISTA GOMES DE SOUZA (Externo)
Dr. EVERTON DO NASCIMENTO ALENCAR (Interno)
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Dr. ADRIANO CESAR DE MORAIS BARONI (Interno) (Suplente)

RESULTADO FINAL: Aprovação

OBSERVAÇÕES:

Nada mais havendo a ser tratado, a Presidente declarou a sessão encerrada e agradeceu a todos pela presença.

Assinaturas

Documento assinado eletronicamente por **Teofilo Fernando Mazon Cardoso**, **Professor do Magisterio Superior**, em 08/12/2023, às 07:49, conforme horário oficial de Mato Grosso do Sul, com fundamento no § 3º do art. 4º do Decreto nº 10.543, de 13 de novembro de 2020.

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DEDICATÓRIA

Dedico este trabalho a minha querida família, que tanto admiro e com muito carinho e apoio, não mediram esforços para que eu chegasse até esta etapa de minha vida, e a todos os que me ajudaram ao longo desta caminhada.

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Muito Obrigada!

“Conhecimento não é aquilo que você sabe, mas o que você faz com aquilo que você sabe.”

Aldous Huxley

RESUMO

Sulfametazina (SMZ), trimetoprima (TMP) e doxiciclina (DOXI), é uma associação de farmácos utilizada no tratamento de infecções intestinais e respiratórias que afetam aves e suínos. O objetivo deste estudo foi desenvolver e validar um método simples, sensível e rápido para determinação simultânea de SMZ, TMP e DOXI em formulação veterinária por cromatografia líquida de alta eficiência de acordo com as diretrizes do Guia de validação e controle de qualidade analítica: fármacos em produtos para alimentação e medicamentos veterinários, RDC 166/2017 e guias internacionais: Conferência Internacional em Harmonização (ICH) e Associação Internacional de Químicos Analíticos Oficiais (AOAC). A separação foi realizada em coluna analítica Macherey-Nagel (MN) C8 (4 mm x 125 mm, 5 µm), com vazão de 0,5 mL min^{-1} e detecção em 268 nm, 270 nm e 350 nm, para SMZ, TMP e DOXI, respectivamente. Todas as medições foram realizadas em acetonitrila:água (45:55 v/v; pH 3,0 ajustado com ácido fosfórico). As curvas analíticas foram lineares (r> 0,9997) na faixa de concentração de 5,0 a 35,0 µg mL^{-1} para SMZ, 1,0 a 7,0 µg mL^{-1} para TMP e 7,0 a 13,0 µg mL^{-1} para DOXI. O método mostrou-se preciso, com coeficientes de variação abaixo do limite máximo de 2,0%, robusto, sem influência significativa das variações utilizadas na análise, exato (recuperação >99%) e seletivo, na avaliação da interferência dos adjuvantes. O estudo de degradação forçada foi realizado em condições hidrolíticas alcalinas, ácidas, neutras e oxidativas, e em condições fotolíticas, neste foram identificados sete produtos de degradação. Na estabilidade acelerada, a amostra foi analisada durante 6 meses (40 ± 2ºC), ao final do estudo não foram observadas grandes variações na análise organoléptica e no pH. As termoanálises verificaram a compatibilidade fármaco-fármaco, foi possível obter informações parciais sobre a estabilidade térmica dos fármacos SMZ, TMP e DOXI em combinação com misturas binárias e ternárias e na amostra comercial. As curvas termoanalíticas mostraram redução na estabilidade térmica dos fármacos quando combinados em misturas binárias e ternárias quando comparados à análise de fármacos individuais. Além disso, a amostra comercial foi mais estável que a mistura ternária de SMZ, TMP e DOXI. Portanto, o método desenvolvido mostrou-se adequado para análises rotineiras de controle de qualidade para determinação simultânea de SMZ, TMP e DOXI em formulações farmacêuticas.

Palavras-chave: Sulfametazina; Trimetoprima; Doxiciclina; Validação analítica; HPLC.

ABSTRACT

Sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY), is a combination of drugs used in the treatment of intestinal and respiratory infections that affect poultry and pigs. The aim of this study was develop and validate a simple, sensitive and fast method for the simultaneous determination of SMZ, TMP and DOXY a veterinary formulation by high performance liquid chromatography according to the guidelines of the Validation and analytical quality control guide: pharmaceuticals in food products and veterinary medicines, RDC 166/2017 and international guides: International Conference on Harmonization (ICH) and International Association of Official Analytical Chemists (AOAC). The separation was performed on a Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm), with a flow rate of 0.5 mL min^{-1} and detection at 268 nm, 270 nm and 350 nm, for SMZ, TMP and DOXY, respectively. All measurements were performed in acetonitrile:water (45:55 v/v; pH 3.0 adjusted with phosphoric acid). The analytical curves were linear (r> 0.9997) in the concentration range of 5.0 to 35.0 µg mL^{-1} for SMZ, 1.0 to 7.0 µg mL^{-1} for TMP and 7.0 to 13.0 µg mL^{-1} for DOXY. The method proved to be accurate, with coefficients of variation below the maximum limit of 2.0%, robust, without significant influence of the variations used in the analysis, exact (recovery> 99%) and selective, in the assessment of interference from adjuvants. The forced degradation study was carried out under alkaline, acidic, neutral and oxidative hydrolytic conditions, and photolytic conditions, seven degradation products were identified. In accelerated stability, the sample was analyzed for 6 months (40 ± 2ºC), at the end of the study, no major variations were observed in organoleptic analysis and pH. The thermo analyses verified drug-drug compatibility, it was possible to obtain partial information on the thermal stability of SMZ, TMP and DOXY drugs in combination with binary and ternary mixtures and in the commercial sample. Thermoanalytical curves showed a reduction in the thermal stability of drugs when combined in binary and ternary mixtures when compared to individual drug analysis. In addition, the commercial sample was more stable than the ternary mixture of SMZ, TMP, and DOXY. Therefore, the developed method proved to be suitable for routine quality control analyses for the simultaneous determination of SMZ, TMP and DOXY in pharmaceutical formulations.

Keywords: Sulfamethazine; Trimethoprim; Doxycycline; Analytical validation; HPLC.

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DEVELOPMENT AND VALIDATION OF METHOD FOR THE SIMULTANEOUS DETERMINATION OF SULFAMETHAZINE, TRIMETHOPRIM AND DOXYCYCLINE IN VETERINARY FORMULATION USING HPLC

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ABSTRACT

Sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY), is a combination of drugs used in the treatment of intestinal and respiratory infections that affect poultry and pigs. The aim of this study was develop and validate a simple, sensitive and fast method for the simultaneous determination of SMZ, TMP and DOXY a veterinary formulation by high performance liquid chromatography according to the guidelines of the Validation and analytical quality control guide: pharmaceuticals in food products and veterinary medicines, RDC 166/2017 and international guides: International Conference on Harmonization (ICH) and International Association of Official Analytical Chemists (AOAC). The separation was performed on a Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm), with a flow rate of 0.5 mL min^{-1} and detection at 268 nm, 270 nm and 350 nm, for SMZ, TMP and DOXY, respectively. All measurements were performed in acetonitrile:water (45:55 v/v; pH 3.0 adjusted with phosphoric acid). The analytical curves were linear (r> 0.9997) in the concentration range of 5.0 to 35.0 µg mL^{-1} for SMZ, 1.0 to 7.0 µg mL^{-1} for TMP and 7.0 to 13.0 µg mL^{-1} for DOXY. The method proved to be accurate, with coefficients of variation below the maximum limit of 2.0%, robust, without significant influence of the variations used in the analysis, exact (recovery> 99%) and selective, in the assessment of interference from adjuvants. The forced degradation study was carried out under alkaline, acidic, neutral and oxidative hydrolytic conditions, and photolytic conditions, seven degradation products were identified. In accelerated stability, the sample was analyzed for 6 months (40 ± 2ºC), at the end of the study, no major variations were observed in organoleptic analysis and pH. The thermo analyses verified drug-drug compatibility, it was possible to obtain partial information on the thermal stability of SMZ, TMP and DOXY drugs in combination with

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Keywords: Sulfamethazine; Trimethoprim; Doxycycline; Analytical validation; HPLC

1. INTRODUCTION

Veterinary products, as well as pharmaceuticals, are subdivided into therapeutic classes: parasiticides, biologicals (vaccines), treatment of infections, food additives and others (CAPANEMA et al., 2007).

Among the classes, antimicrobials are essential substances for the treatment of bacterial infections in animals, since there are hardly any therapeutic options capable of replacing them for the same function (UNGEMACH MÜLLER-BAHRDT and ABRAHAM, 2006).

Antimicrobials are substances that act by inhibiting the growth or destroying microorganisms and are used in the prevention and treatment of dermal, respiratory, urinary, gastrointestinal infections, otitis, mastitis, wounds and surgical prophylaxis. Among the classes of antimicrobials, the most used are: penicillins, cephalosporins, macrolides, lincosamides, tetracyclines, potentiated sulfas, aminoglycosides and fluoroquinolones (FIGUEIREDO, DIAS and ARRUDA, 2008; BAHR ARIAS et al., 2008).

Sulfamethazine (SMZ), chemically called 4-amino-N-(4,6-dimethylpyrimidin-2-yl)benzene-1-sulfonamide , has a molecular formula $C_{12}H_{14}N_4O_2S$ and a molar mass of 278.33 g mol^{-1} (DRUG BANK, 2023; CHEMICAL BOOK, 2022), is an antimicrobial of the sulfonamide class, used for the treatment of cattle, horses, pigs, poultry, small ruminants and rabbits, and can be administered in drinking water, as a food additive and intravenously. It has been marketed alone and in combination with other antimicrobials, such as other sulfonamides, tylosin, chlortetracycline, and procaine penicillin G (RIVIERE and PAPICH, 2009).

Trimethoprim (TMP) is a drug of the pyrimidine class, with antimalarial and antibacterial activity (DARRELL et al., 1968), chemically known as 2,4-diamino-5-(3',4',5'-trimethoxybenzyl)-pyrimidine, with molecular formula $C_{14}H_{18}N_4O$ and molecular mass of 290.32 g mol^{-1} (MOFFAT, OSSELTON and WIDDOP, 2011). This

drug can be found in combination with several drugs from the sulfonamide class. The TMP-sulfonamide association is used to inhibit folic acid metabolism, interfering with purines and bacterial DNA synthesis (SYKES, 2013).

Doxycycline (DOXY), chemically called 2,4-dibromo-6-[[cyclohexyl (methyl) amino] methyl] aniline, has molecular formula $C_{22}H_{24}N_2O_8$ and molar mass of 462,5 g mol^{-1} (MOFFAT, OSSELTON and WIDDOP, 2011). It is a broad-spectrum drug of the tetracycline class, used concomitantly for rickettsiae, sexually transmitted infections, Lyme disease, ophthalmic infections, anthrax, alternative treatment for selected infections when penicillin is contraindicated, acute intestinal amebiasis, prophylaxis of malaria traveler's diarrhea and severe acne (FDA, 2018).

The association of SMZ, TMP and DOXY is used in veterinary medicine for the treatment of intestinal and respiratory infections that affect poultry and swine. For poultry, its use is indicated for chronic respiratory disease in chickens, *Mycoplasma gallisepticum*, *Escherichia coli*, enteritis, *Salmonella* spp and *E.coli*, in swines it is used for atrophic rhinitis, *Bordetella bronchiseptica*, enteritis and *Campylobacter jejuni* (BULÁRIO VETERINÁRIO, 2023).

This association must be administered orally, mixed with feed, for birds in dosages: therapeutic: 105 mg/kg of live weight or curative: 1 kg/ton. of feed, for 3–5 consecutive days, and for pigs: therapeutic: 34 mg/kg of live weight or curative: 1 kg/ton. of feed for 3–5 days. In the case of sows, to prevent contamination of newborn piglets, the recommended dosage is: 2 kg/ton. of feed, for 2 weeks before calving and one week after calving (BULÁRIO VETERINÁRIO, 2023).

When administering this medication together with feed, the industry recommends that this should be the animals' only source of food as long as symptoms persist. Furthermore, the water supply must be maintained during treatment to avoid kidney problems. The treatment must be suspended before the slaughter of animals for human consumption, 5 days before for poultry and 21 days for pigs, and cannot be administered to laying birds during the period of egg production for human consumption (BULÁRIO VETERINÁRIO, 2023).

Despite its clinical importance, analytical methodologies for the simultaneous determination of this association in veterinary drugs were not found in the literature.

Therefore, the objective of this study was to develop and validate an analytical method for the simultaneous determination of the association of SMZ, TMP and DOXY in a formulation for animal use by High Performance Liquid Chromatography (HPLC).

2. LITERATURE REVIEW

2.1 VETERINARY MARKET

Agribusiness in Brazil is in constant growth, qualification and innovation. In this context, the veterinary product and drug industries have invested in new technologies for the development of products that reduce environmental damage, with efficiency and profitability in their use (CALARGE, SATOLO and SATOLO, 2007).

Brazil represents one of the five largest veterinary markets in the expanding world. The increase in exports of veterinary products boosted sanitary inspection, generating more and more criteria for internal or external commercialization. In addition, ranchers are increasingly aware of the need to keep herds healthy, often through health programs (CAPANEMA et al., 2007).

The high demand for veterinary products is observed due to the high number of industries installed in Brazil, especially in the state of São Paulo. These industries can be large, such as pharmaceutical chemical multinationals, operating in the global market and in constant development and innovation, or small, Brazilian industries that serve the local market and specialize in order to meet local needs and niche markets (CAPANEMA et al., 2007).

In Brazil, the animal health segment has shown growth in the market over the years, and may exceed the 10 billion mark. Despite the advances made in this sector, in 2022 the industry suffered from the effects of the pandemic, with rising costs of inputs and packaging (SINDAN, 2023).

In 2022, the Brazilian animal health market achieved an increase of around 10%, especially in products intended for dogs, cats and ruminants (SINDAN, 2023). Regarding the representativeness of revenue by species in 2021, ruminants represent 51%, followed by dogs and cats, which increased their participation to 25%, due to increased care for these animals and greater added value and technology in production. Poultry represented 15%, pigs 13% and horses 2% (SINDAN, 2023).

As for therapeutic classes, antiparasitics accounted for 26% of the market, maintaining their highest consumption performance since 2015. Next are biologicals (22%), antimicrobials (14%), therapeutics (14%), supplements/additives (13%) and others (11%) (SINDAN, 2023).

According to the report of antimicrobials sold or distributed for use in production animals, published by the Center for Veterinary Medicines of the Food and Drug

Administration (FDA) in December 2022, the classes approved for use in food- producing animals for sale were: aminocoumarins, aminoglycosides, amphenicols, cephalosporins, diaminopyrimidines, fluoroquinolones, glycolipids, ionophores, lincosamides, macrolides, orthosomycins, penicillins, pleuromutilins, polymyxins, polypeptides, quinoxalines, streptogramins, sulfonamides and tetracyclines (FDA, 2022).

Regarding domestic sales and distribution of antimicrobials approved for use, it was observed that tetracyclines represent the largest volume of these sales in the domestic market (3.916.864 kg in 2021) around 65% however there was a reduction in sales of 1% in the years 2020 and 2021. Penicillins was the second class with the highest number of sales (10%), but a 19% reduction in sales was observed. Macrolides represented 9%, aminoglycosides 6%, sulfonamides 5%, lincosamides 3%, cephalosporins and fluoroquinolones for less than 1% (FDA, 2022).

Among the routes of administration of these antimicrobials for veterinary use in the year 2021, the most chosen route was feed (64%), followed by water (30%), injectable (5%), oral or topical (1%) and intramammary (< 1%) (FDA, 2022).

As for use in species, 41% were intended for use in cattle, about 42% were intended for use in pigs, about 11% were intended for use in turkeys, about 3% were intended for use in chickens, and an estimated 3% intended for use in other species/unknown (FDA, 2022).

2.2 ANTIMICROBIALS IN VETERINARY MEDICINE

Antimicrobials are drugs widely used in veterinary medicine, indicated for the treatment of bacterial infections, prevention, promotion of animal growth and well-being, through use in the diet at subtherapeutic doses, and disinfection of equipment and materials that come into contact with animal products in the food industry (SPINOSA, GÓRNIAK and BERNARDI, 2006; MARSHALL and LEVY, 2011; RIBEIRO, CORTEZI and GOMES, 2018).

According to Van Boeckel et al. (2015), it is estimated that the consumption of antimicrobials by animals in the world in the year 2030 will be 105.596 tons, and almost double this value in Brazil, Russia, India, China and South Africa, which will lead to decrease in the number of bacteria sensitive to these drugs.

The prescription of antimicrobials by veterinarians must be carried out exclusively for animals that are under their direct care. Veterinarians and producers must follow policies and protocols for prevention, health and treatment programs to maintain animal

health. These must be in accordance with the principles of rational use, good agricultural practices and quality assurance programs (WHO, 2000; WHO, 2017).

In practice, these drugs can be administered by producers and veterinarians to animals, individually, in cows, calves and pigs, or in groups, through water, feed or by injection in hatcheries and feedlots (MCEWEN, 2006).

The amount of antimicrobials to be used in livestock is associated with the number of animals, the production system, the risk of developing diseases and the ability to acquire these drugs by animal producers (PAGE and GAUTIER, 2012).

For the treatment of diseases, antimicrobials represent only a part of the total expense, since there are other costs associated with diagnosis, procedures, involvement of other animals by the disease, in addition to the loss of efficiency in the production of animals that survive the disease (GUSTAFSON and BOWEN, 1997).

The use in improving production was discovered in the 1940s, since then several antimicrobials are being used to contribute to increased weight gain, feed efficiency or change other parameters related to animal production (GUSTAFSON and BOWEN, 1997; MAIORKA et al., 2001), which result in a decrease in the microbiota of the intestinal tract, greater availability of nutrients for the animal and less substrate for the growth of bacteria (HARDY, 2002).

The administration of subtherapeutic doses to animals to promote growth and prevent disease has been rapidly increasing, as a result of the high production of meat and animal products for human consumption. Although this practice has generated positive economic results, mainly in Southeast Asia and China, it is contributing to the growth of bacterial resistance and threatening access to effective antimicrobials for the treatment of diseases in animals (VAN BOECKEL et al., 2015).

According to Chantziaras et al. (2014), the use of antimicrobials in swine, poultry and cattle is correlated with bacterial resistance to these drugs. Bacterial resistance to antimicrobials is a major problem faced by humans and animals (BARBOSA and LEVY, 2000), as in addition to leading to therapeutic failure or the need to use more expensive and toxic drugs, it can also increase the frequency, duration or severity of an infection (MCEWEN, 2006).

Bacterial resistance can be mediated by multiple mechanisms. There are three pathways that bacteria can use to develop antimicrobial resistance (HARDY, 2002):

1. Bacteria become resistant to antibiotics in the animal and this is transferred to humans through consumption of contaminated food.

2. Resistance develops in the bacteria present in the animal, and may not be pathogenic for humans, but transfer resistance to bacteria present in humans.

3. By the presence of antimicrobial residues in food that gives human bacteria the ability to develop resistance to antimicrobials.

As for the progress of resistance to antimicrobials, it is necessary to regulate the consumption of these drugs, especially in food-producing animals. In addition, it is essential that there are laws that reduce the use, disease prevention and use of alternative sources of antimicrobials (LALOUČKOVÁ and SKŘIVANOVÁ, 2019).

Therefore, it appears that these drugs are essential for use in veterinary medicine, however, when not used therapeutically, they contribute to the dissemination of resistant bacteria. For these reasons, this is the therapeutic class most discussed by scientists, producers and government agencies (MARSHALL and LEVY, 2011; RATH, MARTINEZ-MEJIA and SCHRODER, 2015).

In addition to bacterial resistance, another problem related to the administration of veterinary drugs is environmental contamination, which can cause damage to the terrestrial and aquatic environment, contaminating soils, rivers and lakes, in addition to being toxic to fish, birds and plants, due to the high intrinsic toxicity of its degradation products (MARGALIDA et al., 2014; AGA et al., 2016).

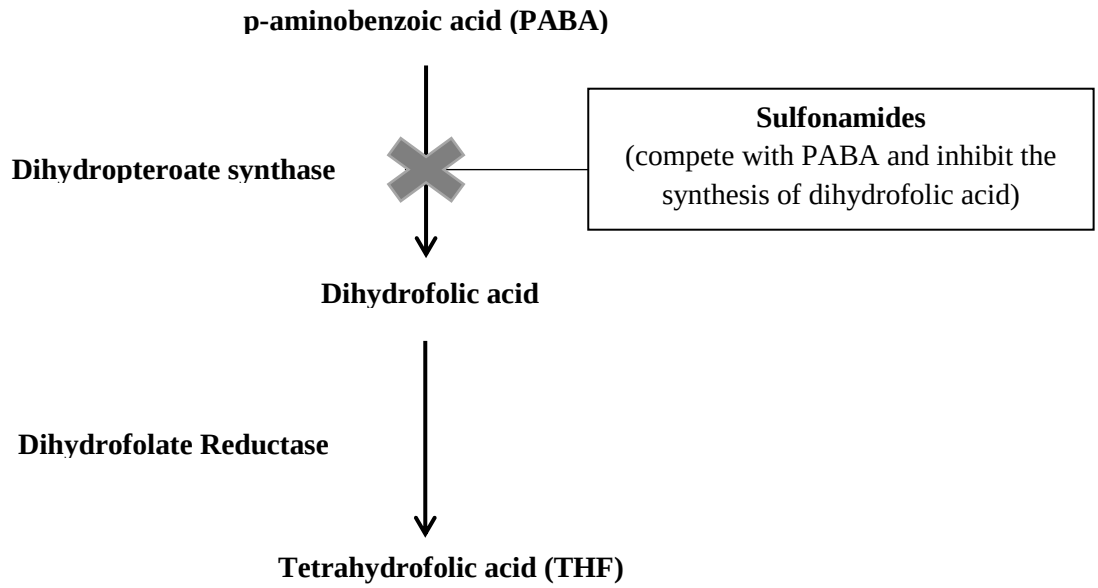
2.2.1 Sulfonamides

Sulfonamides, also known as sulfas, are bacteriostatic antimicrobials produced by chemical synthesis, used in human and veterinary medicine. In 1935, sulfanilamide, the active pharmaceutical ingredient of Prontosil®, was the first antimicrobial agent of this class that was shown to be effective in clinical use to cure bacterial infections in human beings (BILL, 2016; OVUNG and BHATTACHARYYA, 2021).

These compounds have a broad spectrum of action against Gram-positive and Gram-negative bacteria, and are used for the treatment of bacterial diseases caused by *E. coli*, *S. pneumoniae* and *H. influenzae*. They can be found in combination with the medications trimethoprim, pyrimethamine and ormetropime, with the aim of expanding the spectrum of action (LINDSAY et al., 1996; OVUNG and BHATTACHARYYA, 2021). They are structural analogues of para-aminobenzoic acid (PABA), which act by blocking the synthesis of tetrahydrofolic acid, through competition with dihydropteroate synthetase, the enzyme responsible for the incorporation of folic acid, essential for the

survival of bacteria that need to synthesize it (OVUNG and BHATTACHARYYA, 2021) (Figure 1).

Figure 1. Mechanism of action of sulfonamides.



Source: Adapted from Levinson, 2016.

After oral administration, they are well absorbed and have high bioavailability (CHRISTAKI, 2017). They bind strongly to proteins, metabolization occurs via the liver by acetylation, oxidation or glucuronidation, oxidation being responsible for several adverse events. After biotransformation, the metabolites are excreted renally (KESTER, KARPA and VRANA, 2012).

Regarding elimination time, sulfonamides are classified into: short action ($t_{1/2}$ < 8h), medium action ($t_{1/2}$ = 8–16 h), prolonged action ($t_{1/2}$= 17–48 h) and ultra-long-lasting ($t_{1/2}$ > 48h) (CHRISTAKI, 2017).

Side effects associated with sulfonamides are: nausea, diarrhea, cholestatic hepatitis, rash, exfoliative dermatitis, Stevens-Johnson syndrome, neutropenia and thrombocytopenia (KESTER, KARPA and VRANA, 2012).

The chemical structure of sulfonamides has the formula R-SO_2NHR, in which the functional groups are linked to the aromatic ring or to a heteroatom, which can be oxygen or nitrogen (CARTA, SUPURAN and SCOZZAFAVA, 2014).

In relation to the Biopharmaceutical Classification System (BCS), most sulfonamides belong to class II – low solubility and high permeability (RAHIMPOUR, ACREE and JOUYBAN, 2021).

Since the discovery of sulfanilamide, several other drugs of the class have been synthesized from the original compound, such as: sulfacetamide, sulfachloropyridazine, sulfadiazine, sulfamerazine, sulfadimethoxine, sulfadoxine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfapyridine, sulfathiazole, sulfasalazine, sulfisoxazole (PANG, 2018). These were produced with new properties, among them: increased antibacterial potency, decreased toxicity, high or low solubility and prolonged duration of action (GREENWOOD, 2010).

In veterinary medicine, sulfonamides are widely used for the prophylaxis of infectious diseases, as they are effective, stable, easy to administer in feed and water, low cost and display broad spectrum of action. In addition, they are bactericidal when used in combination with synergistic antimicrobials (RIVIERE, 1991; GUAN et al., 2017).

Its metabolization is slow and long-lasting in the body, which can result in residues in cattle and poultry, which can be accumulated in the human body due to the ingestion of products of animal origin that contain excess sulfonamide residues, causing various toxicological effects, such as alterations in the hemopoietic system, allergy and cancer (CHEN, 2002).

Among the sulfonamides, the most used in veterinary treatments are: sulfadimethoxine, sulfathiazole, sulfamerazine, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfacetamide and sulfasalazine (BARAN et al., 2011).

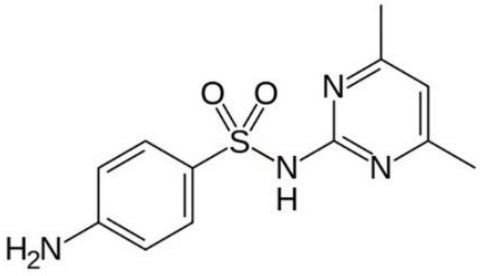
Sulfamethazine, also known as sulfadimidine, has been used since the 1950s to treat respiratory diseases and promote animal growth. It is found in combination with trimethoprim and sulpha drugs such as sulfadiazine and sulfamerazine (IARC, 2001). In cattle and pigs it is used for the treatment of bacterial enteritis, pneumonia, pododermatitis and intestinal coccidiosis (PAPICH and RIVIERE, 2013).

2.2.1.1 Sulfamethazine (SMZ)

SMZ ($C_{12}H_{14}N_4O_2S$) (Figure 2), CAS 57-68-1, is chemically known as 4-amino-N-(4,6-dimethylpyrimidin-2-yl)benzene-1-sulfonamide. Its physical and chemical properties are: white to yellowish-white powder, which may darken on exposure to light, practically odorless, very slightly soluble in water and ether, soluble in acetone, slightly soluble in alcohol, molar mass 278.33 g mol^{-1}, pKa= 7.4 and 2.65, melting point between 197°C and 198.5°C (DRUG BANK, 2023; CHEMICAL BOOK, 2022).

It is synthesized through the reaction of 4-acetylaminobenzenesulfonylchloride with 2-amino-4,6-dimethylpyrimidine, which is synthesized by acetylacetone condensation with guanidine, followed by hydrolysis of the acetylamino group using a base (LAVANYA, 2017).

Figure 2. Chemical structure of sulfamethazine (CAS 57-68-1).



Like sulfonamides, SMZ acts by interfering with the biosynthesis of folic acid in bacterial cells, by competing with PABA for the incorporation of the folic acid molecule. By replacing PABA and preventing the formation of folic acid, necessary for the synthesis of bacterial DNA, bacterial cell multiplication is prevented. Therefore, only organisms that synthesize their own folic acid are sensitive to SMZ (SATOSKAR, 1988).

In the literature, its use is reported for a wide variety of animals, including: cattle, horses, pigs, birds, small ruminants and rabbits, and can be administered in drinking water, as a food additive and intravenously. It has been marketed alone and in combination with other antimicrobials, such as other sulfonamides, tylosin, chlortetracycline, and procaine penicillin G (RIVIERE, PAPICH, 2009).

SMZ has intermediate to long action with pharmacokinetic parameters, dose and frequency of administration, maintenance of the therapeutic index in tissues and body fluids, variable according to the species (RIVIERE, 1997). It is well absorbed after oral administration and extensively metabolized, mainly by the acetylation reaction. The mean plasma half-life is 1.5-5 h, varying with the acetylator status of the drug (GREENWOOD, 2010). Most of the SMZ is excreted in the urine (MITCHELL, PAULSON and ZAYLSKIE, 1986; GOODMAN, SANFORD and GILMAN, 2001).

SMZ has activity against several pathogenic bacteria, with a spectrum of action similar to that of the sulfonamide group, however its potency is relatively low when compared to sulfadiazine, sulfamethoxazole and sulfisoxazole, with the minimum inhibitory concentration (MIC) for bacteria: *Staphylococcus aureus* 32 mg/L – >64 mg/L resistant, *Streptococcus pyogenes* 1 mg/L – 64 mg/L, *Streptococcus pneumoniae* 4 mg/L

– 64 mg/L, *Enterococcus faecalis* >64 mg/L resistant, *Haemophilus influenzae* 8 mg/L L – 16 mg/L, *Neisseria gonorrhoeae* 16 mg/L – >64 mg/L resistant, *Neisseria meningitidis* 0.5 mg/L – 8 mg/L, *Escherichia coli* 16 mg/L – 64 mg/L, *Klebsiella pneumoniae* 64 mg/L – >64 mg/L resistant, *Pseudomonas aeruginosa* >64 mg/L resistant (GREENWOOD, 2010).

SMZ is used as a broad-spectrum antimicrobial to treat or prevent infections caused by susceptible organisms. Treated infections may include pneumonia, coccidiosis, soft tissue infections, and urinary tract infections (PAPICH, 2011).

Recommended SMZ dosages for cattle are: for treatment of pneumonia and other infections 220 mg/kg starting dose, followed by 110 mg/kg after 24h, 237 mg/kg starting dose, followed by 119 mg/kg after 24h in ointments or drinking water and 350-400 mg/kg as a single dose sustained-release bolus. For the treatment of pigs the recommended dosage is: initial dose of 237 mg/kg, followed by 119 mg/kg after 24 hours in powder soluble as ointment or in drinking water. In small animals, cats and dogs, the initial dose should be 100 mg/kg, followed by 50 mg/kg after 12 hours (PAPICH, 2011).

For the determination and quantification of SMZ in biological fluids, food, feed and medication, different analytical methods have been described in the literature, such as: colorimetry, biosensors, immunoassay, immunoenzymatic assay, microbiological diffusion assay, microtiter plate assay, gas chromatography, spectrometry, atomic emission, electron capture detection, thin layer chromatography, high performance thin layer chromatography, tandem mass liquid chromatography, high performance liquid chromatography (HPLC) and fluorescence, fluorimetry (IARC, 2001).

HPLC is one of the most used and applied methodologies in research on the separation of environmental, pharmaceutical, biological and food matrices (PAPICH and RIVIERE, 2013).

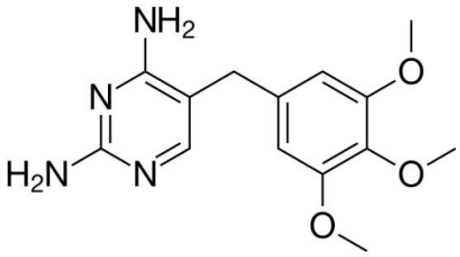
2.2.2 Trimethoprim (TMP)

TMP is a drug that was first described in 1962, which belongs to the pyrimidine class, with antimalarial and antibacterial activity (DARRELL et al., 1968).

It is known chemically as 2,4-diamino-5-(3',4',5'-trimethoxybenzyl)-pyrimidine, with molecular formula $C_{14}H_{18}N_4O$ and molecular mass of 290,32 g mol^{-1}. It is presented as a white to cream crystalline powder, odorless, which melts at 199ºC to 203ºC, with a pKa of 7.16. Its solubility is: very slightly soluble in water, soluble in benzyl alcohol,

slightly soluble in chloroform and in methanol, slightly soluble in alcohol and in acetone, practically insoluble in ether and in carbon tetrachloride (Figure 3) (MOFFAT, OSSELTON and WIDDOP, 2011; CHEMICALIZE, 2023).

Figure 3. Chemical structure of trimethoprim (CAS 738-70-5).



In the Biopharmaceutical Classification System (BCS), it is classified in class 2 - low solubility and high permeability (LINDENBERG, KOPP and DRESSMAN, 2004).

It is a structural analogue of pteridine of dihydrofolic acid, with bactericidal action, which inhibits, through competition, dihydrofolate reductase and, consequently, the production of THF from dihydrofolic acid (MASTERS et al., 2003).

Acts synergistically with sulfonamides to inhibit folic acid metabolism, interfering with purines and bacterial DNA synthesis. Resistance of the trimethoprim-sulfonamide combination occurs by plasmid-mediated production of altered dihydrofolate reductase or dihydropteroate synthetase with reduced binding affinities, overproduction of dihydrofolate reductase or PABA by bacteria, and reduced bacterial permeability to trimethoprim and sulfonamides (SYKES, 2013).

TMP is lipid-soluble at physiological pH and has a large volume of distribution (100–120 L), has a half-life of about 15 hours, is metabolized hepatically and excreted by the kidneys (CHRISTAKI, 2017).

Clinically, the combination of trimethoprim-sulfonamide is used due to its broad spectrum of action, for the treatment of gram-positive and gram-negative bacterial infections, and some protozoan infections. In veterinary medicine, the formulation of ormethoprim-sulfadimethoxine, similar to the combination of trimethoprim-sulfonamide, is used for the same clinical indications (SYKES, 2013).

Adverse effects are similar to those of sulfonamides, including pruritic skin rashes, gastrointestinal discomfort, hematological abnormalities and fever, with rash and fever being more common in patients with HIV (KESTER, KARPA and VRANA, 2012).

2.2.3 Tetracyclines

Chlortetracycline was the first tetracycline discovered in 1945 by Benjamin Duggar, obtained by the product of natural fermentation of bacteria, *Streptomyces aureofaciens*, present in soils. This discovery aroused interest in conducting research to obtain new tetracyclines. Between 1950 and 1970, other tetracyclines were developed, of natural or semi-synthetic origin, which in the same period became the most used antimicrobials in the United States (PEREIRA-MAIA et. al., 2010).

This class of antimicrobials has a broad spectrum of action for Gram-positive and Gram-negative bacteria, chlamydia, mycoplasma, rickettsia, parasites and protozoa. One of its characteristics is the absence of side effects of great importance, which led to its extensive use for the treatment of humans and animals. In addition, they are also used for the prevention of malaria caused by mefloquine-resistant *Plasmodium falciparum*, and in some countries, such as the United States, they are added at subtherapeutic levels to animal feeds to promote growth (CHOPRA and ROBERTS, 2001).

After the discovery of the first generation of tetracyclines, obtained by fermentation, in the period from 1950 to 1970, two other generations were produced by synthesis, with better pharmacological and toxicological properties (PEREIRA-MAIA et al, 2010; RAMACHANDERAN and SCHAEFER, 2021).

The tetracyclines described in the literature are: oxytetracycline, lymecycline, doxycycline, minocycline, chlortetracycline, tetracycline, methacycline, desmethylchlortetracycline, rolitetracycline and clomocycline (CHOPRA and ROBERTS, 2001). Among the tetracyclines, the most active when compared are glycylcyclines, minocycline and doxycycline, as they have a greater lipophilic character (PEREIRA-MAIA et al, 2010).

Among the tetracyclines used in clinical routine, tigecycline cannot be administered orally, while oxytetracycline, doxycycline and minocycline are administered by oral and parenteral routes. (PEREIRA-MAIA et al, 2010).

Tetracyclines act by inhibiting the 30S ribosomal subunit, making it difficult for the aminoacyl-tRNA to bind to the site in the mRNA-ribosome complex. In this way, the process is interrupted and the bacterial cell is no longer able to maintain adequate functioning and is not able to grow or replicate. The reduction in efficacy and resistance to tetracyclines occurs through mechanisms of alteration of ribosomal protection proteins or efflux pumps (SHUTTER e AKHONDI, 2023).

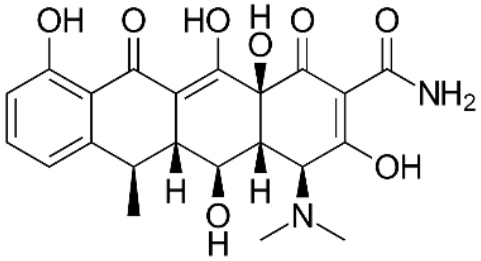
As they are low-cost medicines with a broad spectrum of action, they are widely used for prophylaxis and treatment of infections in humans and animals, and as animal growth promoters (CHOPRA and ROBERTS, 2001). In veterinary medicine, tetracyclines are used to treat gastrointestinal diseases, respiratory, skin and systemic infections, sepsis and diseases of the locomotor organs and genitourinary tract. (MICHALOVA, NOVOTNA and SCHLEGELOVA, 2004).

The use in agriculture as a growth promoter and treatment of humans and animals are the main causes of tetracycline pollution in the aquatic environment, due to the toxic effects of tetracyclines on aquatic organisms that disturb the balance, causing dysbiosis. Additionally, tetracycline residues have also been found in soil, surface waters, marine environments, sediments, and biota samples. These residues can accumulate throughout the food chain and be toxic to the microbial community, increasing antimicrobial resistance, representing a public health problem due to the various harmful effects of tetracycline contamination on the ecological system (AMANGELSIN et al., 2023).

2.2.3.1 Doxycycline (DOXY)

DOXY is a broad-spectrum antimicrobial with bacteriostatic action, discovered in the early 1960s by Pfizer Inc., which was sold under the brand name Vibramycin® (TAN et al., 2011) (Figure 4).

Figure 4. Chemical structure of doxycycline (CAS 24390-14-5).



It is a yellow crystalline powder, very slightly soluble in water, sparingly soluble in ethanol, practically insoluble in chloroform and ether, freely soluble in dilute acids and alkali hydroxides (MOFFAT, OSSELTON and WIDDOP, 2011), pKa of 3.27 and 8.33, and melting point of 206°C to 209°C (CHEMICAL BOOK, 2023). Compared to other tetracyclines, DOXY has lower affinity for metal ions, but is capable of forming chelate complexes (YANG et al., 2015; SMITH and COOK, 2004).

The monohydrate form, is chemically called 2,4-dibromo-6-[[cyclohexyl (methyl) amino] methyl] aniline, has a molecular formula $C_{22}H_{24}N_2O_8$ and a molar mass of 462.5

g mol^{-1}, and the hydrochloride has a molecular formula $C_{22}H_{24}N_2O_8$,HCl,1/2C_2H_5OH,1/2H_2O and molar mass 512.9 g mol^{-1} (MOFFAT, OSSELTON and WIDDOP, 2011). It has high solubility and high permeability, belonging to class I of the Biopharmaceutical Classification System (BCS) (LINDENBERG, KOPP and DRESSMAN, 2004), when it is in the hydrochloride and hyclate salts forms. It is industrially synthesized from chemical modifications of fermentation tetracyclines: oxytetracycline or methacycline (VARDANYAN and HRUBY, 2016).

It has been used in human and veterinary medicine for over 40 years, due to its greater lipid solubility in relation to other tetracyclines, greater volume of distribution and better penetration, even with a high percentage of binding to plasma proteins (80% - 90%) (MONSALVE et. al., 2017).

DOXY has broad bacteriostatic action against Gram-positive and Gram-negative bacteria and some protozoa, such as malaria. It has also been used for other non-infectious diseases, such as severe acne, although part of its effect may be due to antibacterial activity against *Propionibacterium acnes*, periodontitis, rosacea, bullous dermatoses, neutrophilic diseases, *Pyoderma gangrenosum*, sarcoidosis, multiple sclerosis, medical therapy of aortic aneurysms, autoimmune diseases such as rheumatoid arthritis and scleroderma, pleurodesis procedures and lymphatic or vascular malformations (HOLMES and CHARLES, 2009).

Among tetracyclines, it has the best permeation through cell membranes, has a prolonged half-life and lower renal toxicity (MAJEWSKI, 2014). Penetration occurs in body fluids and tissues, as it is more lipid-soluble it is easily transported through cell membranes and can be detected in several locations, including lymphatic fluid, peritoneal fluid, colon tissue, prostate tissue and breast milk. The serum half-life is prolonged to 18 to 22 hours and is not altered by renal failure, and peak serum levels occur 2-3 hours after oral administration and 30 minutes after intravenous administration (HOLMES and CHARLES, 2009). It is excreted in bile and feces and to a lesser extent it is excreted in urine by the kidneys (AGWUH and MACGOWAN, 2006).

The toxicity is similar to that of other tetracyclines, with the main adverse events being: gastrointestinal irritation, skin rashes and photosensitization, in addition to being able to cause tooth stains and fatty necrosis of the liver in pregnant women or in patients receiving more than 2 g/day of DOXY (SCHOLAR, 2007).

In the clinical practice of Veterinary Medicine, its use is increasing for the treatment of infections in various animal species, such as ehrlichiosis or respiratory tract diseases

in dogs, pneumonia in cattle and pigs, colibacillosis and psittacosis in poultry (CASTRO et al., 2009).

2.3 QUALITY CONTROL OF PHARMACEUTICAL PRODUCTS

The ineffectiveness of drugs for use in veterinary medicine poses a threat to public health, since the spread and transmission of diseases can occur through the interactions that exist between animals and humans. Thus, it is essential to use quality medicines so that the treatment of animals is safe and effective, thus avoiding the spread and development of zoonoses (SMITH, 2013).

Quality deviations interfere with the effectiveness of the treatment, allow the induction of microbial resistance and generate financial losses in animal production for the owner and for the country (MOTA et al., 2005).

Compliance with good practices in production and use must be carried out for both veterinary medicines and medicines for human use. The lack of minimum quality requirements makes treatment ineffective, influencing the spread of diseases among animals, the presence of residues in food above the maximum permitted limits or the appearance of resistant strains as a result of the administration of subtherapeutic doses (RATH et al., 2015)

Given the importance of drug quality control for the pharmaceutical industry, the development and validation of analytical methods is essential to optimize laboratory resources and meet objectives in the drug production stages so that it meets its specifications and quality attributes (BHAGAT and SAUDAGAR, 2019; DESHMUKH et al., 2019).

To choose the analytical method to be developed, some factors need to be taken into consideration: the objective of the methodology, analysis time, cost, feasibility and ease of execution of the method, reliability of results, reproducibility and adequate instrumentation (BONFILIO, 2009). Furthermore, when developing the method, it is also necessary to study the properties of the drugs, such as: solubility, polarity, pKa and pH (DESHMUKH et al., 2019).

To ensure that the developed method is suitable for use, some validation parameters need to be analyzed: accuracy, precision (repeatability, intermediate precision and reproducibility), specificity, limits of detection and quantification, linearity, stability, robustness and suitability of the system method (BHAGAT and SAUDAGAR, 2019; DESHMUKH et al., 2019).

In view of this, the importance of evaluating the quality of medicines in the pharmaceutical industry in the development and manufacturing processes is highlighted, to guarantee safety and the desired therapeutic effect.

2.3.1. High Performance Liquid Chromatography (HPLC)

Liquid chromatography began in 1950, with the use of columns with irregular particles of 100-200 µm with an efficiency of just 200 plates/15 cm (MALDANER and JARDIM, 2009).

This technique is used to separate components present in a sample, through the process of differential migration in a system composed of two phases (mobile phase and stationary phase) in one direction. (SABIR, MOLOY and BHASIN, 2016).

HPLC is the separation technique most used today for qualitative and quantitative purposes for chemical analysis, as it allows determinations with good sensitivity, in different separation modes: normal, reverse, size exclusion, ion exchange and bioaffinity (TONHI et al., 2002; MALVIYA et al., 2010).

In reversed-phase chromatography, separation depends on the interaction of molecules in a solution between the mobile phase and the stationary phase (PRATHAP et al., 2013). In this case, the stationary phases are nonpolar, and columns with chains of nonpolar groups C8 and C18 are the most used (TONHI et al., 2002).

There are several solvents that can be used in reversed-phase HPLC, the most commonly used being acetonitrile and methanol. In addition to these, water can also be used to compose the mobile phase, with acids or salts that help separate the components of a sample (THAMMANA, 2016). As for the sample elution mode, it can be in isocratic or gradient mode (PRATHAP et al., 2013; THAMMANA, 2016), and must be selected based on the nature and hydrophobicity of the analytes (DESHMUKH et al., 2019).

During the development of an analytical method with HPLC, some parameters must be taken into account (specificity, accuracy, precision, limit of detection, linearity), as in any other method to be validated (PRATHAP et al., 2013; THAMMANA, 2016).

In addition to choosing the column and mobile phase, another point to be decided is the detectors and quantification to be carried out, always taking into account the best mobile phase, pH, detector, column, wavelength for method development, as these factors have fundamental role in the selectivity of separation (PRATHAP et al., 2013; THAMMANA, 2016).

The detector is an important factor to be considered, as its selection must take into account the chemical nature of the analyses, interferences, detection limits and detector cost. The UV-visible detector is considered versatile as it offers high sensitivity in routine analyzes of quantification and identification of impurities (VIDUSHI and MEENAKSHI, 2017).

HPLC is one of the most used techniques in chemical analysis as it presents several advantages: simultaneous analysis, high resolution, high sensitivity, good repeatability, small sample quantity and easy sample fractionation and purification (VIDUSHI and MEENAKSHI, 2017).

Therefore, it is possible to verify that HPLC is the most used separation technique in the pharmaceutical industry for production monitoring, stability assessment and quality control of finished products, as it is a highly selective, sensitive, simple, versatile technique with diverse applications. in the pharmaceutical, forensic, environmental and clinical areas (RODRÍGUEZ, PEZZA and PEZZA, 2016; VIDUSHI and MEENAKSHI, 2017).

Some studies found in the literature demonstrated that HPLC can be used successfully to determine sulfamethazine (Table 1) and doxycycline (Table 2) in pharmaceutical matrices.

Table 1. Analytical methods using high-performance liquid chromatography for determining sulfamethazine in pharmaceutical matrices.

Matrice	Column	λ (nm)	Mobile Phase/Solvents	Flow (mL min^{-1})	Linearity range	References
Veterinary formulations	Kromasil C18 (150 mm x 4.6 mm; 5 µm)	263, 210 e 248	40 mM NaH2PO4 buffer solution, containing 10 mM NaClO4 (pH 3.0) – acetonitrile (65:35)	1.5 ml/min	0.2–11 mg/L	NEVADO, PENALVO and BERNARDO, 2000.
Tablets	150 mm × 4.6 mm	272	Methanol: acetic acid (9:1 v/v)	1,0 mL/min	0,20 a 0,8 g/L	HUNG et al., 2007.
Pharmaceutical formulation	LiChrosphere C18 (125 mm × 4.6 mm; 5 µm) and C18 precolumn (4.0 mm × 4.0 mm; 5 µm)	203	1400 mL de água, 400 mL de ACN e 2 mL de trietilamina	-	50 a 250 µg mL^{-1}	MAMANI et al., 2008.
Drug	Kinetex biphenyl (2.1 mm x 50 mm; 5 µm) coupled to biphenyl precolumn (2.0 x 4.0 mm)	-	0.1% formic acid in water (phase A) and Acetonitrile with 0.1% formic acid (phase B) 0 - 1 min 100% A 1 - 11 min 70% A 11 – 13 min 5% A 13 - 17 min 100% A 17 min - 24.0 min 100% A	400 µL/min	SMZ: 50 – 400 mg/kg TMP: 50 – 200 mg/kg	PATYRA et al., 2018.
Drug and feed	Zorbax Eclipse XDB C18 (150 mm ×4.6mm, 5µm)	260	0.05 M sodium dodecyl 0.02 M sulfate/phosphate buffer (pH 3) and 6% propan-2-ol mixture	0.6 mL/min	0.004–0.4 mg/mL	PATYRA and KWIATEK, 2021.

Table 2. Analytical methods using high-performance liquid chromatography for determining doxycycline in pharmaceutical matrices.

Matrice	Column	λ (nm)	Mobile Phase/Solvents	Flow (mL min^{-1})	Linearity range	References
Bulk samples, tablets, capsules and suspensions	Hamilton PRP-I (250 x 4.6 mm, 10 µm)	254	Mixing 750 mL of water, 60 mL of tetrahydrofuran, 100 ml of 0.2 M phosphate buffer (pH 8.0), 50 mL of 0.02 M tetrabutylammonium hydrogen sulphate (TBA) solution and 10 ml of 0.1 M sodium edetate (EDTA).	1.0 mL/min	30-50 µg	DIHUIDI et al., 1985.
Pharmaceutical mixing	Hamilton PRP-1	272	Phosphate-citrate buffer 7x10-2 mol L^{-1} (pH 5): 2-propanol: tetrahydrofuran: dichloromethane (81:11:7:1, v/v/v/v)	0.8 mL min^{-1} 1.0 mL min^{-1}	-	YASIN and JEFFERIES, 1988.
Standard	PLRP-S 250 mm x 4.6 mm	254	2-methyl-2-propanol(6.0 g)-0.2 M phosphate buffer pH 8.0 (10.0 mL)-0.02 M tetrabutylammonium sulphate pH 8.0 (5.0 ml)-0.1 M EDTA pH 8.0 (1.0 mL)-water (up to 100 mL)	1.0 mL min^{-1}	16-24 µg	HOOGMARTENS et al., 1989.
Standard	RSiL CN 250 mm x 4.6 mm	280	Tetrahydrofuran-dimethylformamideacetic acid-water-EDTA, 72:10:16:2:0.0015 (v/v/v/v/m)	2.0 mL min^{-1}	16-24 µg	HOOGMARTENS et al., 1989.
Commercial samples and standards	Poly (styrene-divinylbenzene) (250 mm x 4.6 mm)	254	2-methyl-2-propanol: potassium phosphate buffer 2x10^{-1} mol L^{-1} (pH 8): hydrogenated tetrabutylammonium sulfate 2x10^{-2} mol L^{-1} (pH 8), 1x10^{-2} mol L^{-1} EDTA (pH 8): water (5.8: 10:5:10:69,2, v/v/v/v/v)	1.0 mL/min	8-12 µg	NAIDONG et al, 1990.
Standard and bulk powder	C18 PLRP-S (PSDVB) (250 mm × 4.6 mm, 5 µm) PM-C18 (150 mm x 4.6 mm)	280	Acetonitrile-0.02 M sodium perchlorate, pH 2.0	-	-	BRYAN and STEWART, 1993.
Standard and bulk powder	PLRP-S (25 cm x 4.6 mm) PM-C18	280	Acetonitrile-sodium perchlorate (pH 2.0, 0.02 M) (25:75, v/v).	1.0 ml min^{-1}	-	BRYAN and STEWART, 1994.

	(15 cm x 4.6 mm)					
Bulk powder, tablets and capsules	C18 (300 mm x 4.6 mm)	-	Potassium acid phosphate $5x10^{-2}$ mol L^{-1} (pH 2.5): ACN (84:16, v/v)	1.0 mL min^{-1}	0.1–50 ng ml^{-1}	KAZEMIFARD and MOORE, 1997.
Bulk powder and tablets	PGC (100 mm x 4.6 mm)	268	Potassium phosphate buffer $5x10^{-2}$ mol L^{-1} (pH 2):acetonitrile (40:60, v/v)	1.0 mL min^{-1}	5-50 µl ml^{-1}	MONSER and DARGHOUTH, 2000.
Pharmaceutical products	Phenomenex Luna C8 (250×4.6 mm, 5 µm) with a Phenomenex C8 4×10 mm guard column	350	Acetonitrile:water: perchloric acid (HClO4) (26:74:0.25) adjusted to pH 2.5 with 5 M sodium hydroxide	1.0 mL/min	3–60 µl/mL	SKÚLASON, INGÓLFSSON and KRISTMUNDSDÓTTIR, 2003.
Standard and commercial samples	XTerra RP-18 (25 cm × 4.6 mm; 5 µm)	280	Acetonitrile – 0.2 M TBA pH 7.0 – 0.3 M EDTA pH 7.0 – water (130:350:350:170, v/v/v/v)	1.0 mL min^{-1}	12.5–1000 µg mL^{-1}	YEKKALA et al., 2003.
Drug mixture for food	Phenomenex Synergi Polar-RP 80-A (150 mm x 2 mm)	346	Oxalic acid (0.02 M; pH 2.5)-acetonitrile 82:18 (v/v)	0.3 mL/min	0.0125, 0.025, 0.05, 0.075 and 0.1 mg/mL	FIORI et al., 2005.
Substances from different manufacturers	C18	280	0.05mol/L diammonium oxalate: N, N-dimethylformamide: 0.2 mol/L diammonium hydrogen phosphate: methanol (50:36:4-10)	0.9mL/min	2.500-249.956 µg/mL	WANG, ZHANG and ZENG, 2006.
Bulk, tablets, and capsules	µ-Bondapak C8 column (150 mm x 4.6 mm, 5 µm)	350	Acetonitrile–water–THF (29.5:70:0.5, v/v/v), adjusted to pH 2.5 with 1.0M HCl	1.0 mL/min	5–100 mg/L	INJAC, DJORDJEVIC-MILIC, SRDJENOVIĆ, 2007.
Standard and tablets	Zorbax SB C18 (250 mm × 4.6 mm)	230	A: 10 mM potassium phosphate buffer (pH 5.1)/methanol 95:5 (v/v) B: 10 mM potassium phosphate buffer (pH 5.1)/methanol, 5:95 (v/v).	0.8 mL/min 1.2 mL/min	Nine standard solutions from 10 to 150% of the test concentration (0.1 mg/mL)	GAUDIANO et al., 2008.
Capsule	Phenomenex Luna C18 (250 mm × 4.6 mm 5 µm)	245	20 mM potassium dihydrogen phosphate, pH 6–acetonitrile in ratio of (1:1, v/v)	1.0 mL min^{-1}	1–21 and 1–100g ml^{-1}	HADAD, EL-GINDY and MAHMOUD, 2008.

Drug	Lichrosorb RP-8 (250 mm x 4.6 mm, 10 µm)	350	Methanol, acetonitrile and 0.010 M aqueous solution of oxalic acid (2:3:5, v/v/v).	1.25 ml/min	25.2–252.0 µg mL^{-1}	MITIC et al., 2008.
Tablets	250 mm × 4.0 mm, 5.0 µm	325	Acetonitrile-potassium dihydrogenorthophosphate buffer (pH 4.0), 40:60 (v/v)	1.0 mL/min	30-300 µg/ mL	RAMESH et al., 2010.
Mixtures and pharmaceutical formulations	Discovery C18 (250 mm x 4.6 mm, 5 µm)	277	A: methanol –0.03M potassium dihydrogen phosphate buffer – triethylamine (30:69.7:0.3, v/v/v), adjusted with 1 M orthophosphoric acid to a pH of 6.6+0.1 B: acetonitrile	1.5 mL/min	2.5–550 µg/ mL	DARWISH et al., 2013.
Tablets	Zorbax C8 (250 mm x 4.6 mm, 5 µm)	293	20mM Potassium dihydrogen ortho phosphate (pH 6, adjusted with triethylamine): Acetonitrile (60:40 % v/v)	1.0 mL/min	10-50 µg/mL	DEOKATE, NAWALE and SALVI, 2013.
Tablets	CN Luna (250 mm ×4.6 mm, 5 µm)	360	Water + 0.1% TFA-acetonitrile + 0.1% TFA, 60:40 (v/v)	1.0 mL/min	50-100 µg/mL	KOGAWA and SALGADO, 2013.
Capsules	C18 (250 mm × 4.6 mm, 5 µm	260	Methanol and 0.06 M sodium dihydrogen phosphate (65 : 35, v/v) adjusted to pH 4.5	1.0 mL min^{-1}	5–30 µg mL^{-1}	WALASH, IBRAHIM and ABASS, 2013.
Tablets	Waters Sunfire C8 (250 mm ×4.6 mm, 5 µm)	400	30 volumes of potassium dihydrogen phosphate buffer (50 mM) with triethylamine and 70 volumes of methanol	0.85 mL/min	200-700 µg/mL	DHAL et al., 2015.
Bulk and pharmaceutical dosage form	Hypersil BDS C18 column (250 mm ×4.6 mm, 5µm)	260	Buffer:Acetonitrile (55:45 v/v)	1.0 mL/min	12.5 – 75 µg/mL	SRILEKHA, PAVANI and SREEDHAR, 2015.
Pharmaceutical bulk and dosage forms	Perfectsil Target ODS column (125 mm x 4 mm, 3-5µm)	269	Methanol-50 mM ammonium acetate buffer (0.1% v/v trifluoroacetic acid and 0.1% v/v triethylamine, pH 2.5) (50:50 v/v)	0.8 mL/min	25-500 µg/mL	POURMOSLEMI et al., 2016.
Tablets	C18 Luna	275	Water + 0.5 % acetic acid and ethanol (40:60, v/v)	0.8 mL min^{-1}	20–200 µg mL^{-1}	GHIDINI, KOGAWA and SALGAGO, 2018.

	(250 mm x 4.6 mm, 5.0 µm)					
Suppositories and tablets	Waters Acquity BEH C18 (2.1 mm × 50 mm, 1.7 µm)	270	75 mM ammonium acetate, 4 mM EDTA (pH 8.8) and acetonitrile (97:3)	1.2 mL/min	50-150 µg/mL	MOHAMMAD et al., 2018.
Tablets	Kromasil C18 (150 mm × 4.6 µm, 5 µm)	250	Aqueous formic acid (0.1%), methanol and acetonitrile	1.0 mL/min	1-100 µg/mL	TEGEGNE et al., 2021
Pharmaceutical formulations	Zorbax SB column (150 mm × 4.6 µm, 5 µm)	350	A: 0.1% formic acid in ultrapure water B: acetonitrile	1.5 mL	10–100 µg/mL	BECZE et al., 2022.
Pharmaceutical forms	Phenomenex Luna C18 (50 mm × 4.6 mm, 3 µm)	269	Water:acetonitrile: perchloric acid (75:25:0.2, v/v)	0.7 mL/min	0.25–50, 0.1–30, 0.2–35, 0.5–50, and 0.6–20 µg/mL	MAHMOUD et al., 2023.
Bulk and parenteral dosage forms	Waters XBridge BEH C8 (150 mm × 4.6 mm, 3.5 µm)	270	A: phosphate buffer (pH 8.5, 25 mM potassium phosphate, 2 mM ethylenediaminetetraacetic acid, and 0.5 mL of triethylamine). B: methanol	1.7 mL/min	Doxycycline: 0.5–150 µg/mL 4-epidoxycycline 0.5–18 µg/mL	PIPPALLA et al., 2023.

3. MATERIAL AND METHODS

3.1 RAW MATERIALS, COMMERCIAL SAMPLE AND SOLVENTS

The drugs SMZ (purity 100%) and TMP (purity 99.24%) were purchased from the pharmaceutical laboratory FURP and DOXY (purity 100%) was purchased from the distributor Organic Compounding, used as standard reference substances were accompanied by the certificate of analysis of the Providers.

The commercial sample used during the development and validation of the analytical method was Doxigram ST® (SMZ 20.0 g + TMP 4.0 g + DOXY 10.0 g every 100 g, donated by Evance Saúde Animal).

The solvents used were: ultrapure water, obtained in the Milli-Q Plus® system and acetonitrile (analytical grade) from the brands JT Baker®, Merck® and Tedia®.

3.2 THIN LAYER CHROMATOGRAPHY (TLC)

To perform the TLC, due to the impossibility of acquiring pharmacopeial standards, the raw materials of SMZ, TMP and DOXY were compared with the commercial sample (Doxigram ST®).

The raw material solutions were prepared at a concentration of 1.0 mg mL^{-1}. The stationary phase used was silica gel 60 F_{254} Merck®, in aluminum plates (chromatoplates) measuring 6 x 3 cm.

To elute the prepared solutions, 37 mobile phases were tested, containing combinations of solvents: ethyl acetate, acetonitrile, acetic acid, water, butanol, chloroform, dichloromethane, ether, and methanol, in different combinations and proportions. The eluents and developer used in the analysis of raw materials and commercial samples studied by TLC are described in Table 3.

The applications of the solutions were carried out with the aid of a glass capillary, with three touches being applied to each solution. After elution and drying of the plates, they were analyzed in a dark chamber with UV light 254 and 365 nm (U.V. SL – 204 Solab®) and the retention factors (Rf) of the raw materials and commercial sample were calculated according to the following formula:

$$\mathrm{Rf} = \frac{\mathrm{Da}}{\mathrm{Dmf}}$$

On what:

Da= distance covered by the sample in cm

Dmf= distance traveled by the mobile phase in cm

Table 3. Mobile phases and revealer used in the analysis of raw materials of sulfamethazine, trimethoprim, doxycycline and commercial sample by thin layer chromatography.

Mobile Phase	Revealer
Acetonitrile:methanol (6:4, v/v)	
Acetonitrile:chloroform (8:2, v/v)	
Acetonitrile:chloroform:methanol (4:4:2, v/v/v)	
Acetonitrile:chloroform:methanol (5:3:2, v/v/v)	
Acetonitrile:chloroform:methanol (6:3:1, v/v/v)	
Acetic acid:butanol (5:5, v/v)	
Acetic acid:butanol (7:3, v/v)	
Acetic acid:water:butanol (1.7:1.7:6,6, v/v/v)	
Acetic acid:water:butanol (1:2:7, v/v/v)	
Acetic acid:water:butanol (1:1:8, v/v/v)	
Acetic acid:water:butanol (1:2:7, v/v/v)	
Acetic acid:water:butanol (1:3:6, v/v/v)	
Acetic acid:water:butanol (1:4:5, v/v/v)	
Acetic acid:water:butanol (2:1:7, v/v/v)	
Acetic acid:water:butanol (3:2:5, v/v/v)	
Acetic acid:water:butanol (3:1:6, v/v/v)	
Acetic acid:water:butanol (4:3:3, v/v/v)	UV Light (254 nm)
Acetic acid:water:butanol (4:4:2, v/v/v)	
Acetic acid:methanol:ethyl acetate (1:6:3, v/v/v)	
Acetic acid:methanol:ethyl acetate (4:3:3, v/v/v)	
Acetic acid:methanol:ethyl acetate (3:2:5, v/v/v)	
Chloroform:methanol (7:3, v/v)	
Chloroform:methanol (5:5, v/v)	
Chloroform:methanol (1:9, v/v)	
Chloroform:methanol (3:7, v/v)	
Chloroform:acetonitrile:methanol (1:4:4, v/v/v)	
Chloroform:acetonitrile:methanol (2:2:6, v/v/v)	
Chloroform:acetonitrile:methanol (1:1:8, v/v/v)	
Dichloromethane:methanol:water (6:3:1, v/v/v)	
Ether:methanol (2:8, v/v)	
Ether:methanol (4:6, v/v)	
Ether:methanol (6:4, v/v)	

Methanol:acetic acid (9:1, v/v)
Methanol:ethyl acetate:acetic acid (8:1.5:0.5, v/v/v)
Methanol:ethyl acetate:acetic acid (4:5:1, v/v/v)
Methanol:acetonitrile:acetic acid (8:1.5:0.5, v/v/v)
Methanol:acetonitrile:acetic acid (6:3:1, v/v/v)

3.3 SPECTROSCOPY IN THE ULTRAVIOLET (UV) REGION

Spectroscopy in the ultraviolet region was performed using a ThermoScientific Evolution 60® UV/VIS spectrophotometer. The raw materials SMZ, TMP and DOXY were prepared using solvent acetonitrile:water (45:55 v/v). Absorption spectra were read in the wavelength range between 200 and 400 nm. The spectra obtained were then compared with those already described in the scientific literature.

3.4 SPECTROSCOPY IN THE INFRARED (IR) REGION

Spectroscopy in the infrared region was carried out using a ThermoScientific IR spectrometer, model Nicolet IS5®, using the Attenuated Total Reflection (ATR) technique and the iD3® accessory with Germanium crystal. The spectra were obtained in the range of 4000-500 cm^{-1} with a resolution of 4 cm^{1} and were subsequently interpreted and compared with the literature.

3.5 ASSESSMENT OF THERMAL BEHAVIOR: THERMOGRAVIMETRY (TG), DERIVED THERMOGRAVIMETRY (DTG) AND DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The evaluation of the thermal behavior was carried out through thermoanalytical techniques, with the drugs SMZ, TMP and DOXY. The TG/DTG curves were obtained with the aid of the thermoanalyzer system TGA-Q50 from TA Instruments®, using a platinum crucible, nitrogen atmosphere with a flow rate of 40.0 mL min^{-1}, heating rate of 10ºC min^{-1} to 600ºC. The curves were determined by the tangent, by the instant at which the straight curve suffered a deviation.

The DSC curves of SMZ, TMP and DOXY were obtained in an aluminum crucible covered with a perforated lid, using the DSC-Q20 equipment with a TA Instruments® RCS-90 refrigeration system in a nitrogen atmosphere with a flow rate of 60.0 mL min^{-1} and heating rate of 10°C min^{-1} in the temperature range from -50 to 250°C.

3.6 VALIDATION OF THE ANALYTICAL METHODOLOGY

The analytical parameters of linearity, limit of detection (LD), limit of quantification (LQ), precision, robustness, accuracy, selectivity were evaluated for validation of spectrophotometric methods according to the validation and analytical quality control guide: pharmaceuticals in food products and veterinary medicines (BRASIL, 2011) and RDC 166/2017 (BRASIL, 2017) and guides Internacional Conference on Harmonization (ICH, 2005) and Association of Official Analytical Chemists International (AOAC, 2005).

The results were analyzed statistically using Microsoft Excel® (Microsoft, Washington-USA) and OriginPro 9.0® (OriginLab, Northampton-UK).

3.6.1 Compliance of the Chromatographic System and Optimization of the Analytical Method

To develop the method, the following columns were tested: C18 AcclaimTM120 ThermoScientific® (4.6 x 250 mm, 5 µm), C18 Zorbax Agilent® (4.6 x 150 mm, 5 µm), C18 Kromasil® (4.6 x 100 mm, 5 µm), C18 InertSustain GL Sciences® (4.6 x 150 mm x 5 µm) e C8 InertSustain GL Sciences® (4.6 x 100 mm x 3 µm), C8 Macherey-Nagel (MN)® (4 x 125 mm, 5 µm) with different flows, compositions and proportions of mobile phases, with: methanol and water at pH 3.0, adjusted with phosphoric acid (50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 82:18, 84:16, 85:15, 45:55, 40:60 v/v); acetonitrile and water at pH 3.0, adjusted with phosphoric acid (30:70, 40:60, 45:55, 50:50, 55:45, 60:40, 70:30, 80:20 v/v) and acetonitrile, methanol and water at pH 3.0, adjusted with phosphoric acid (5:45:50, 5:50:45, 10:50:40, 10:60:30 v/v).

The choice of the best chromatographic condition was carried out through the analysis of the parameters: retention time, peak purity, number of theoretical plates, resolution and asymmetry factors, and repeatability, according to the recommendations of the validation guides of chromatographic methods.

3.6.2 Equipment and Chromatographic Conditions

The method was developed and validated in an Ultimate 3000 high-performance liquid chromatograph (Thermo Fisher Scientific, USA), equipped with a diode array detector (DAD) in the 200 to 400 nm range and a quaternary pump.

The chromatographic separation occurred in the isocratic mode at a flow rate of 0.5 mL min^{-1} on an Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm). The mobile phase was composed of acetonitrile and water (45:55 v/v), pH adjusted to 3.0 with phosphoric acid. Detections were performed at 268 nm for SMZ, 270 nm for TMP and 350 nm for DOXY. The injection volume was 20 µL and the running time was less than 4 minutes. The analyzes were performed at room temperature (25.0 ± 1.0 ºC).

Furthermore, with the chromatograph's diode array detector (DAD) and the aid of the Chromeleon® 7.1 program, it was possible to determine the purity of the peaks of each drug by superimposing the UV spectra extracted from the chromatographic peaks.

3.6.3 Preparation of Standard Stock Solutions and Commercial Sample

The standard SMZ, TMP and DOXY stock solutions at 50 µg mL^{-1} were prepared separately in acetonitrile and homogenized in an ultrasonic bath for 15 minutes. Subsequently, each standard solution was diluted using acetonitrile:water (45:55, v/v, pH 3.0) in different concentrations for validation of the analytical method were prepared, as described below.

Standard stock solutions were prepared weighing analytically 10 mg of each standard reference substances (SMZ, TMP and DOXY) that were transferred separately to 10 mL volumetric flasks, the volume was completed with acetonitrile, obtaining solutions at 50 µg mL^{-1} of each drug. Then, these solutions were taken to the ultrasonic bath for 15 minutes.

50 mg of the commercial sample Doxigram ST® was transferred to a 100 mL volumetric flask, the volume was completed with acetonitrile. Then the solution was placed in an ultrasonic bath for 15 minutes. From this solution, a 5 mL aliquot was taken, which was loaded into a 50 mL volumetric flask, which was completed with acetonitrile. This solution was then placed in an ultrasonic bath for 15 minutes. The stock solution obtained from the commercial sample was: SMZ 10 µg mL^{-1}, TMP 2 µg mL^{-1} e DOXY 5 µg mL^{-1}.

The solutions were stored under refrigeration at a temperature of 2ºC to 8ºC and storage period of forty-eight hours. Daily, before each test, the standard solutions were removed from the refrigerator to balance the temperature, and then the samples were diluted to be injected into HPLC. The stability of the solutions was evaluated by analyzing

the standard solutions according to the determination of the content and UV spectra of the drugs.

3.7 ANALYTICAL PARAMETERS

3.7.1 Linearity

It was evaluated by building three analytical curves for each drug. The analytical curve for SMZ was obtained in the concentration range of 5.0 to 35.0 µg mL^{-1}, for TMP in the range of 1.0 to 7.0 µg mL^{-1}, and for DOXY in the range of 7.0 at 13.0 µg mL^{-1}. The determinations were carried out in triplicate and the results were submitted to linear regression analysis to obtain the analytical curves, line equations and correlation coefficients for each drug.

3.7.2 Limit of Detection (LD) and Limit of Quantification (LQ)

The LD and LQ of SMZ, TMP and DOXY were determined from three analytical curves obtained for each drug, using the standard deviation of the intercept (SD) and the mean slope (a). Equations (1) were used to calculate LD and LQ:

$$LD = 3{,}3 \text{ x} \frac{\text{SD}}{\text{a}} \qquad LQ = 10 \text{ x} \frac{\text{SD}}{\text{a}} \qquad (1)$$

3.7.3 Precision

The precision of the method was assessed by intra-day (repeatability) and inter-day (intermediate precision) tests. Repeatability was performed by analyzing six determinations of the stock solution of the commercial sample, under the same chromatographic conditions and by the same analyst.

Intermediate precision was performed by analyzing the commercial sample by two analysts on three different days, at the concentrations mentioned above. The relative standard deviation (RSD) was calculated from the results obtained.

3.7.4 Robustness

Robustness was assessed by the Plackett-Burman factorial model, where seven parameters that can interfere with the analytical result were changed, to analyze the influence of these variations. The seven variables selected were: sonication time, flow

and proportion of the mobile phase, acetonitrile supplier, pH of the mobile phase, wavelength and laboratory temperature.

The conditions of the variables were designated by the letters A to G, the numbers 1 to 15 represent the number of experiments, where the level (0) represents the normal conditions of the method, while the levels of (1) and (-1) are respectively, the upper and lower values in relation to normal conditions (0). The tested parameters and levels are shown in Table 4.

Table 4. Selected factors and levels of variation used in robustness, as per the Plackett-Burman factorial model.

Factors	Unit	Limit	Condition varied (-1)	Condition normal (0)	Condition varied (1)
A – Sonification time	min	± 2	8	10	12
B – Mobile phase flow	mL min^{-1}	± 0.02	0.48	0.50	0.52
C – Acetonitrile mark	---	---	JT Baker	Merck	Tedia
D – Mobile phase proportion	%	± 2	43:57	45:55	47:53
E – Mobile phase pH	---	± 0.2	2.8	3.0	3.2
F – Wavelength	nm	± 2	266 268 348	268 271 350	270 273 352
G – Laboratory temperature	°C	± 2	23	25	27

0: condition normal; -1: lower altered condition; 1: superior altered condition.

Robustness was performed in triplicate from injections of sample solutions, containing 10.0, 2.0 and 5.0 μg mL^{-1} of SMZ, TMP and DOXY respectively, following the factorial combinations established in Table 5.

Table 5. Plackett-Burman factorial combination applied in the robustness test.

Factor	Factorial combination														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
A	1	1	1	0	1	0	0	0	-1	-1	-1	0	-1	0	0
B	0	1	1	1	0	1	0	0	0	-1	-1	-1	0	-1	0
C	0	0	1	1	1	0	1	0	0	0	-1	-1	-1	0	-1
D	1	0	0	1	1	1	0	0	-1	0	0	-1	-1	-1	0
E	0	1	0	0	1	1	1	0	0	-1	0	0	-1	-1	-1
F	1	0	1	0	0	1	1	0	-1	0	-1	0	0	-1	-1
G	1	1	0	1	0	0	1	0	-1	-1	0	-1	0	0	-1

A-G: selected factors; 1-15: number of experiments.

The influence of the variations of each parameter in the final result was determined by the average of the results obtained in the assays with the normal parameters in comparison with the average corresponding to the altered parameters, and the effect

generated by each variable, which is the difference between the results obtained in normal conditions and changed parameters.

The deviation produced by each factor was calculated using the Youden and Steiner methodology (1975). Equation (2) demonstrates the evaluation of the effect caused by the change in variable A (sample sonification time) and the other factors were also evaluated by this equation.

$$\sqrt{2S} > |DA|, \quad (2)$$

Where: $S = \sqrt{\frac{2}{7}}(DA^2 + DB^2 + DC^2 + DD^2 + DE^2 + DF^2 + DG^2)$

For a method to be considered robust, the absolute values of the effects must be less than the critical value (√2S) (PEDROSO and SALGADO, 2014).

3.7.5 Accuracy

The accuracy of the method was determined through recovery tests, performed by adding amounts known from standard solutions to commercial sample solutions. Assays were performed separately for each drug in order to contemplate the linear concentration range of the method. In the recovery tests, standard solutions and previously described stock samples, through recovery assays at three concentration levels for each drug in triplicate.

Quantities of the solution prepared with the raw materials of the drugs were added to the stock solutions in order to obtain concentration solutions of 35.0, 45.0 e 50.0 µg mL^{-1} de SMZ; 5.0, 7.0 e 9.0 µg mL^{-1} de TMP; 14.0, 18.0 e 22.0 µg mL^{-1} de DOXY.

The recovery percentages of each drug were calculated using equation 3 (AOAC, 2005):

$$\%R = \left[\frac{Ca - Cna}{Ctp}\right] x\, 100 \quad (3)$$

Where: R: recovery, Ca: drug concentration found in the standard added sample (µg mL^{-1}), Cna: drug concentration found in the standard non-added sample (µg mL^{-1}) and Ctp: theoretical standard concentration added to the sample (µg mL^{-1}).

3.7.6 Selectivity

The selectivity of the method was determined by evaluating the interference of the adjuvants in the wavelength used for the quantitative determination of the drugs present in combination in the commercial sample.

The list of adjuvants in the commercial sample was not obtained due to industrial secrecy, therefore, a placebo solution was prepared containing a mixture of the following adjuvants: lactose (70%), starch (10%) and cellulose (20%). The sample and the placebo solution were prepared in acetonitrile:water (45:55, pH 3.0) (enough quantity for 100 mL), in a similar way to that previously described for the preparation of stock-sample solutions. The commercial sample chromatogram was compared with the placebo chromatogram.

3.8 METHOD APPLICABILITY

The commercial sample Doxigram ST® (composed of SMZ 20.0%, TMP 4.0% and DOXY 10.0%) was analyzed using the proposed analytical method. The sample-stock solutions were prepared as previously described, obtaining the following theoretical concentrations: SMZ 10.0 μg mL^{-1}, TMP 2.0 μg mL^{-1} and DOXY 5.0 μg mL^{-1}.

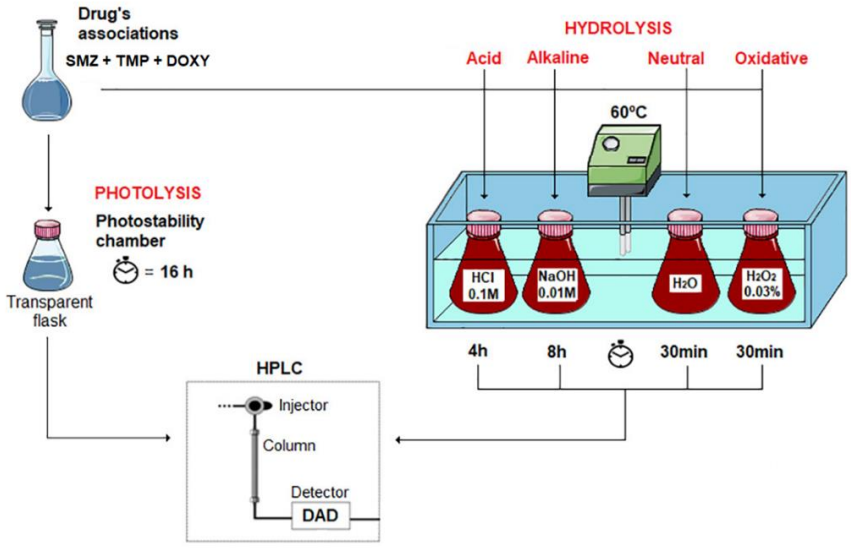
The content of each drug present in the commercial sample was determined under the conditions of the developed analytical method and calculated using the straight line equations of the analytical curves.

3.9 FORCED DEGRADATION STUDY

The forced degradation study was carried out in accordance with the recommendations of the international drug and pharmaceutical stability study guides (ICH, 1996; ICH, 2003) and the experimental protocol (Figure 5) developed by Sversut et al. (2019).

For this, aliquots of the solution of the raw materials in their combined form, that is, SMZ, TMP and DOXY, were subjected to stress conditions, such as acid, alkaline, neutral, oxidative and photolytic hydrolysis. Table 6 shows the degradation conditions that will be tested.

Figure 5. Experimental protocol used to carry out the study of forced degradation.



Source: Adapted from Sversut et al., 2019.

Table 6. Experimental conditions for carrying out the forced degradation study.

Stressful agent	Stress condition	Exposure time
Acid solution	HCl 0,1 M a 60ºC[c]/RT[a]	4 hours
Basic solution	NaOH 0,01 M a 60ºC[c]/RT[a]	8 hours
Oxidative solution	H_2O_2 0,03% (v/v) RT[a]	30 minutes
Heat	H_2O 60ºC[c]	30 minutes
Light	Light UV[b]/RT[a]	16 hours

[a]Room temperature (24 ± 2ºC); [b]exposure light UV (254-365 nm); [c]Water bath.

From a standard stock solution of 50 µg mL^{-1}, solutions were prepared with a mixture of acetonitrile:water (45:55, v/v, pH 3.0), adjusted with phosphoric acid, to reach a final concentration of 10.0 µg mL^{-1} for SMZ, and 2.0 µg mL^{-1} for TMP and 5.0 µg mL^{-1} for DOXY.

The resulting solutions were injected in triplicate and analyzed using the proposed chromatographic method: Macherey-Nagel (MN)® C8 analytical column (4 mm x 125 mm, 5 µm), mobile phase acetonitrile and water (45:55 v/v, pH 3.0), adjusted with phosphoric acid, isocratic mode, flow rate of 0.5 mL min^{-1}.

The percentages of decrease in the content of the analyzed drugs were calculated through the equations of the straight lines obtained from the respective analytical curves, and the purities of the peaks, observed in the spectral profile obtained from the DAD detector.

In order to carry out the forced degradation study, experimental conditions that promoted an average degradation of 10 to 30% for each of the drugs were defined as ideal (WHO, 2009).

3.10 ACCELERATED STABILITY

The study was carried out in accordance with Resolutions (RE) nº 1, of July 29, 2005 and nº 318, of November 6, 2019, which serves as a guide for carrying out stability studies (BRASIL, 2005; BRASIL, 2019). The commercial sample Doxigram ST® was placed in a Petri dish and stored at 40 ± 2ºC for six months, using a NI 1521 oven (NOVA Instruments®), equipped with a temperature controller. The sample was analyzed at 0, 3 and 6 months, through organoleptic evaluation, pH determination and dosage, for comparative purposes.

In the organoleptic evaluation, changes in color or odor were observed during the test. The pH determination was performed in triplicate and at room temperature (25 ± 2ºC) through direct readingof the commercial sample in aqueous solution at 1% (w/v), using MS Tecnopon® mPA210 benchtop pHmeter. The pHmeter was previously calibrated with pH 7 and 4 solutions, as recommended by the equipment.

The assay was performed by high performance liquid chromatography (HPLC), with the previously validated method. The chromatographic conditions were: Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm), mobile phase acetonitrile and water (45:55 v/v, pH 3.0), adjusted with phosphoric acid, isocratic mode, flow rate of 0.5 mL min^{-1}, injection volume of 20 µL, diode array detector set at 268 nm, 270 nm and 350 nm. The sample solutions were prepared in mobile phase, obtaining the theoretical concentrations of: 10.0 µg mL^{-1} for SMZ, and 2.0 µg mL^{-1} for TMP and 5.0 µg mL^{-1} for DOXY.

The areas were registered in triplicate and the drug contents were calculated through equations of the straight lines obtained from analytical curves.

4. RESULTS AND DISCUSSION

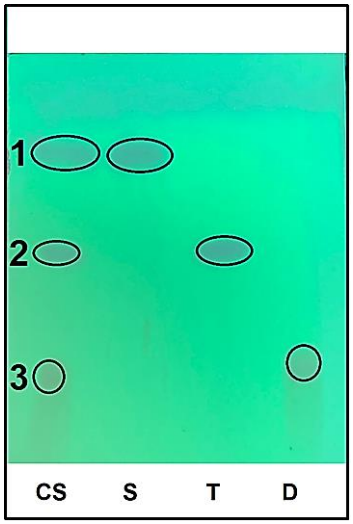
4.1 QUALITATIVE ANALYSIS OF RAW MATERIALS

4.1.1 Thin Layer Chromatography (TLC)

According to the chromatographic profiles obtained and the Rfs values of the drugs, only the mobile phase acetic acid:water:butanol (1:2:7, v/v/v) proved to be suitable for analysis by TLC for drug identification present in the analyzed sample.

Figure 6 presents the chromatograms of the commercial sample containing the associated drugs SMZ + TMP + DOXY and the raw materials of SMZ, TMP and DOXY in stationary phase of silica gel G60 F_{254}, mobile phase composed of acetic acid:water:butanol (1:2:7, v/v/v), using 254 nm UV light as a developer.

Figure 6. Chromatoplates developed in stationary phase of silica gel G60 F254 and mobile phase acetic acid:water:butanol (1:2:7, v/v/v), developed under UV light (254 nm). Chromatographic profiles of commercial sample (CS) and raw materials sulfamethazine (S), trimethoprim (T) and doxycycline (D).



After revealing the chromatographic plate, it was possible to verify in Figure 6 that the stains obtained in the commercial sample (CS) corresponded in position to those obtained with the raw materials with equal Rf values for stains 1 (CS and S) of 0.74 , spots 2 (CS and T) of 0.52 and spots 3 (CS of 0.24 and D of 0.22) (Table 7).

Obtaining this result suggests that the commercial sample and the raw materials of SMZ, TMP and DOXY have the same identity, since the Rf values of the raw materials corresponded to those obtained with the commercial sample, in addition, the profile

chromatogram (Figure 6) did not obtain the appearance of other stains, only that of the analyzed compounds in question.

Table 7. Conditions chosen to perform thin layer chromatography (TLC) of the drugs sulfamethazine, trimethoprim and doxycycline, and results of retention factors.

Drugs	Stationary phase	Mobile Phase	Revelation	Rf
SMZ	Sílica gel G60 F254	Acetic acid:water:butanol (1:2:7, v/v/v)	UV Light (254 nm)	0.74
TMP				0.52
DOXY				0.22

SMZ: sulfamethazine, TMP: trimethoprim, DOXY: doxycycline, Rf: retention factor.

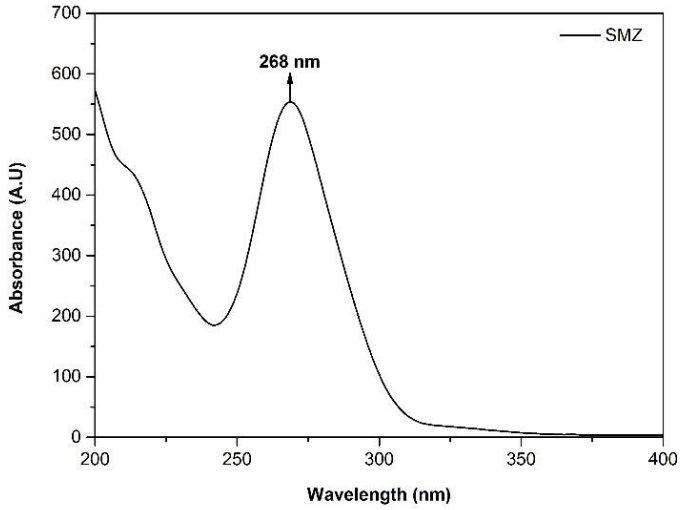
In addition, the method developed using TLC proved to be efficient, accurate, executable and applicable for the analyzed drugs, and can also be used to identify other sulfonamides, screening and quality control of antimicrobials that present in their formulation SMZ, TMP and DOXY.

4.1.2 Spectroscopy in the Ultraviolet (UV) and Infrared (IR) Regions

4.1.2.1 Sulfamethazine (SMZ)

In spectroscopy in the ultraviolet region, SMZ presented a band with maximum absorption at 268 nm, as illustrated in Figure 7.

Figure 7. Absorption spectrum in the UV/Vis region of the raw material sulfamethazine (SMZ) using acetonitrile:water (45:55 v/v) as solvent.



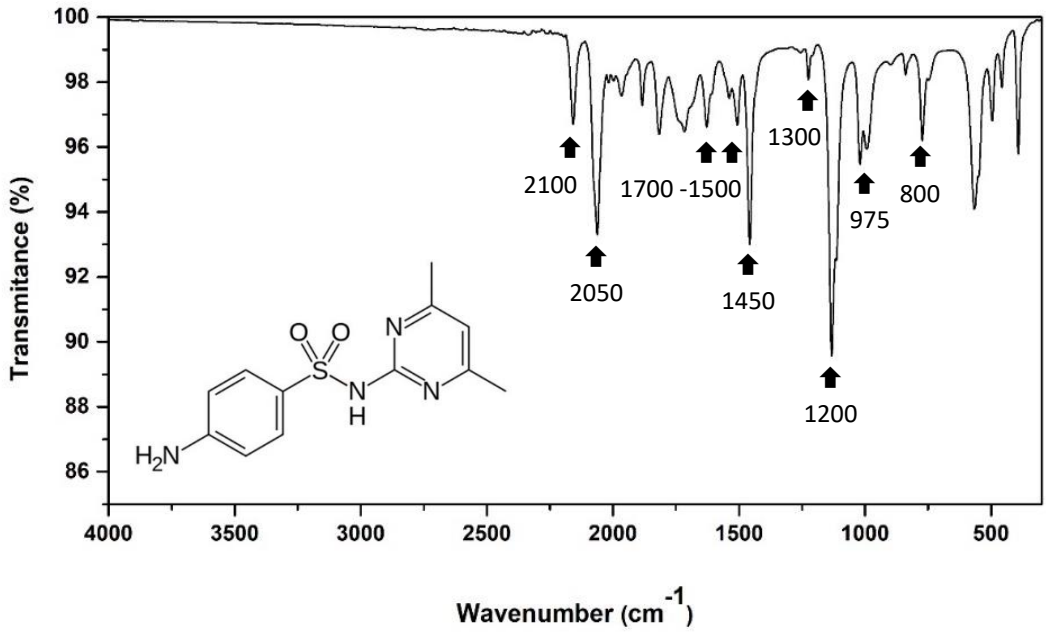
Source: Own author using Origin Pro 9.0

The same spectral profile of the drug SMZ obtained in the UV/Vis region was also found by Nassar et al. (2017) with λmax of 263 nm in aqueous solution, Srinivasan et al.

(2012) with λmax of 241 nm in methanol, Moffat, Osselton and Widdop (2011) with a band with λmax of 301 nm in acidic water and 258 nm in alkaline solution and Lertpaitoonpan, Ong and Moorman (2009) with λmax of 264 nm in 0.01 M $CaCl_2$.

In the IR spectrum of the SMZ molecule (Figure 8), it was possible to verify the presence of functional groups with absorption frequencies characteristic of the drug. The low intensity bands observed at 2100 and 2050 cm^{-1} are characteristic, respectively, of the stretching of the N=C=N bond. Between 1700 – 1500 cm^{-1} two low intensity bands were identified relating to the vibration of the C=C group. The folding of the C-H bond was identified at 1450 cm^{-1}. The band at 1300 cm^{-1} can be attributed to the vibration of the C-N bond and the band at 1200 cm^{-1} to the SO_2 group. Finally, at 975 and 800 cm^{-1}, bands related to the C-H group of the aromatic ring were identified, referring to the bending vibrations of the 1,3,5 substitution.

Figure 8. Absorption spectrum in the infrared region of the raw material sulfamethazine obtained in the range of 4000-500 cm^{-1} with the Attenuated Total Reflection (ATR) technique and the iD3® accessory with Germanium crystal.



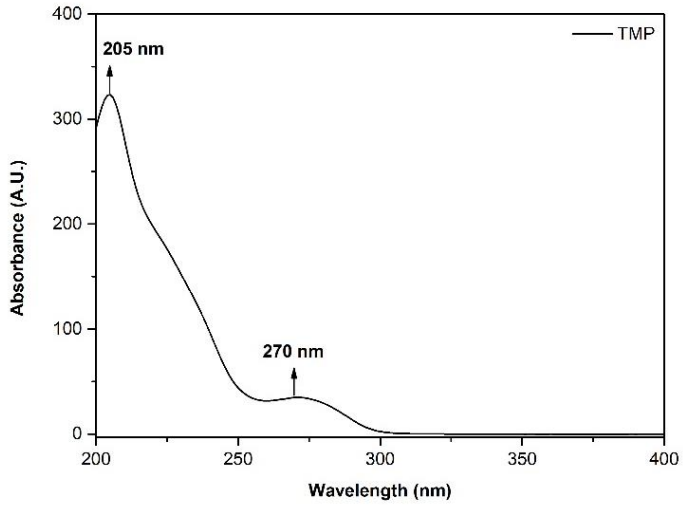
Source: Own author using Origin Pro 9.0

4.1.2.2 Trimethoprim (TMP)

DOXY presented two bands in the spectrum in the UV region with maximum absorption at 205 nm and 270 nm as illustrated in Figure 9. The spectrum obtained from the drug presented the same spectral profile as that found by Moffat, Osselton and Widdop

(2011) with a band with λmax of 271 nm in acidic water and 287 nm in alkaline solution, Ashour et al. (2011), Muchlisyam, Parede and Satiawan (2018) with a band with λmax of 288 nm in alkaline solution.

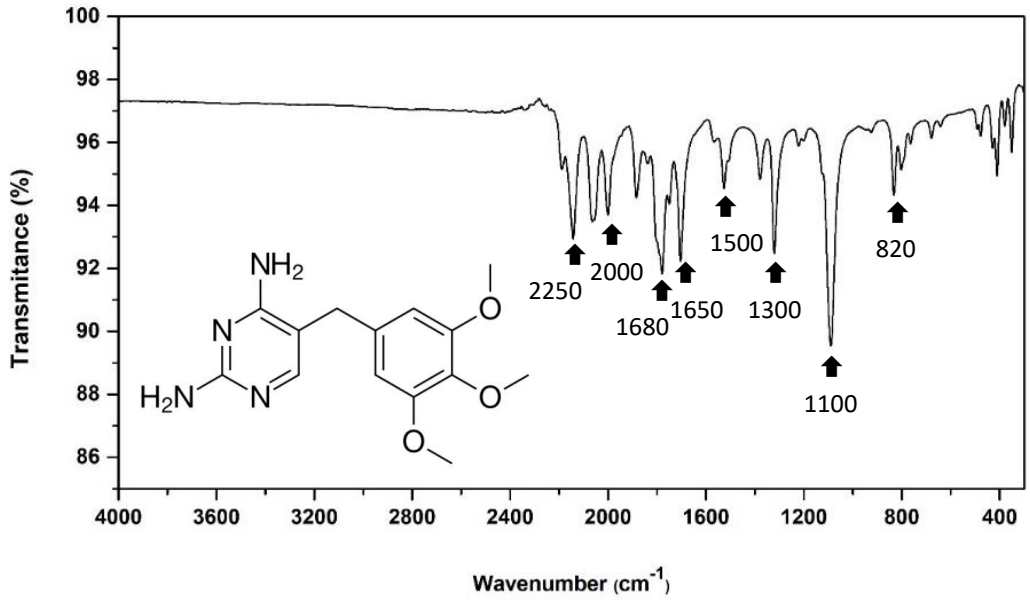
Figure 9. Absorption spectrum in the UV/Vis region of the raw material trimethoprim (TMP) using acetonitrile:water (45:55 v/v) as solvent.



Source: Own author using Origin Pro 9.0

The absorption spectrum in the IR region is shown in Figure 10.

Figure 10. Absorption spectrum in the infrared region of the raw material trimethoprim obtained in the range of 4000-500 cm^{-1} with the Attenuated Total Reflection (ATR) technique and the iD3® accessory with Germanium crystal.



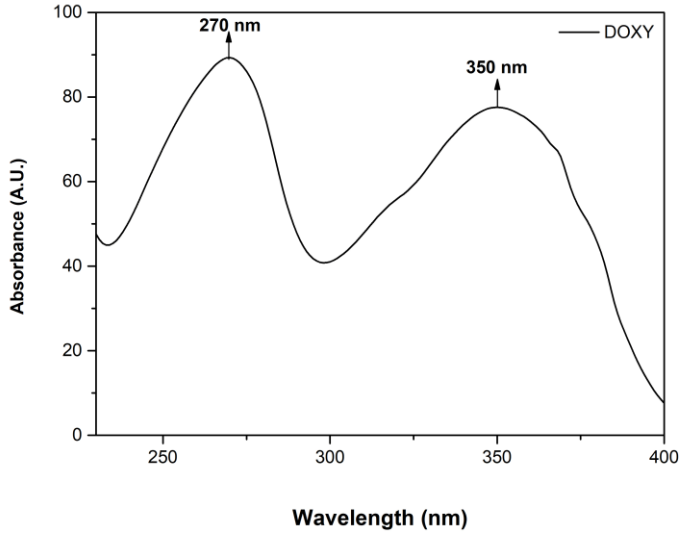
Source: Own author using Origin Pro 9.0

The characteristic bands of the TMP functional groups were identified in the 2250 cm-1 region corresponding to the stretching of the N=C=N group, in the 2000 cm^{-1} region is the stretching vibration of the C=C=N group, at 1680 cm^{-1} the C=N stretching was observed, the C=C group was observed in the bands in the region of 1650 and 1500 cm^{-1}, in the region of 1300 to 1100 cm^{-1} the stretching of the C-O bond was found . Finally, in the region of 820 cm^{-1}, a band corresponding to the C-H bond and folding vibration of the 1,2,3 substitution is noted.

4.1.2.3 Doxycycline (DOXY)

DOXY presented two bands in the spectrum in the UV region with maximum absorption at 270 nm and 350 nm as illustrated in Figure 11.

Figure 11. Absorption spectrum in the UV/Vis region of the raw material doxycycline (DOXY) using acetonitrile:water (45:55 v/v) as solvent.



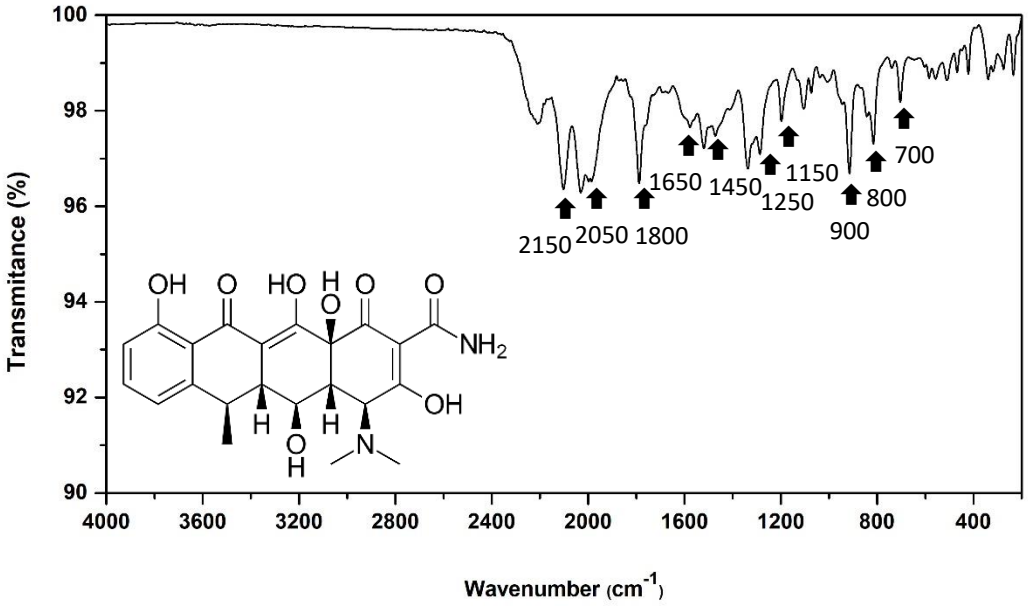
Source: Own author using Origin Pro 9.0

The same spectral profile was also found by Moffat, Osselton and Widdop (2011) with λmax of 269 and 346 nm in acidic water, Victor and Kumar (2011) with λmax of 270 nm and 350 nm, Kogawa and Salgado (2013) λmax of 268 nm in hydrochloric acid and Khampieng, Wnek and Supaphol (2014) with λmax of 351 nm.

In the absorption spectrum in the IR region of DOXY, bands characteristic of the drug's functional groups were found, as shown in Figure 12. The low intensity band observed at 2150 cm^{-1} is characteristic of the C=C=O stretching, while the band at 2050 cm^{-1}, also of low intensity, is attributed to the bending of the C-H bond. The characteristic

vibration band of the bond between carbon and carbonyl oxygen (C=O) is observed at 1800 cm^{-1}. At 1650 cm^{-1} there is a band characteristic of N-H folding and at 1450 cm^{-1} a band characteristic of C-H folding. The band at 1250 cm^{-1} refers to the bending of the O-H bond and at 1150 cm^{-1} a band characteristic of C-O stretching. At 900 cm^{-1} the observed band is characteristic of the C=C fold. Lastly, the two bands at 800 and 700 cm^{-1} are characteristic of the folding of the C-H bond of the aromatic ring.

Figure 12. Absorption spectrum in the infrared region of the raw material doxycycline obtained in the range of 4000-500 cm^{-1} with the Attenuated Total Reflection (ATR) technique and the iD3® accessory with Germanium crystal.



Source: Own author using Origin Pro 9.0

4.2 THERMOGRAVIMETRY (TG), DERIVED THERMOGRAVIMETRY (DTG) AND DIFFERENTIAL SCANNING CALORIMETRY (DSC)

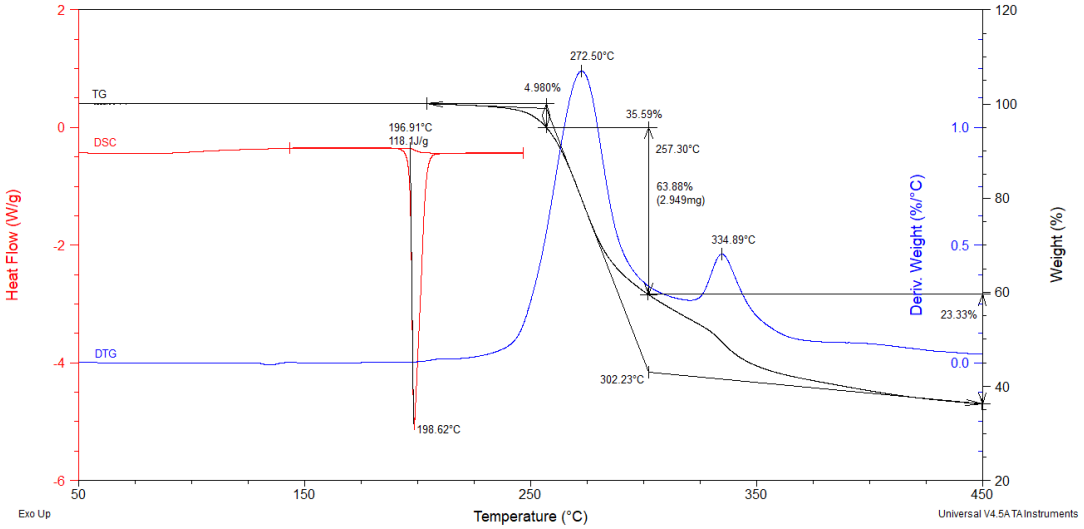
4.2.1 Sulfamethazine (SMZ)

The SMZ TG/DTG curves (Figure 13) demonstrated that the substance was thermally stable up to a temperature of 204.06°C and that there was no loss of characteristic moisture mass.

Two mass loss events occurred: between 257.30 and 302.23°C with a mass variation of 63.88%, and between 330.26 and 345.85°C with a mass variation of 12.53%. At the end of the heating process, the mass loss was 67.87%, leaving 32.13% of inorganic residue. In the DTG curve, two exothermic peaks were observed at 272.50°C and 334.80°C.

The DSC curve showed a fine endothermic peak between 189.69°C and 210.86°C, with an enthalpy of 118.1 J g^{-1} and 196.1°C, corresponding to the melting of the SMZ. The calculated T*onset* was 198.62°C, corroborating the melting point data of 198°C to 199°C described by Moffat, Osselton and Widdop (2011).

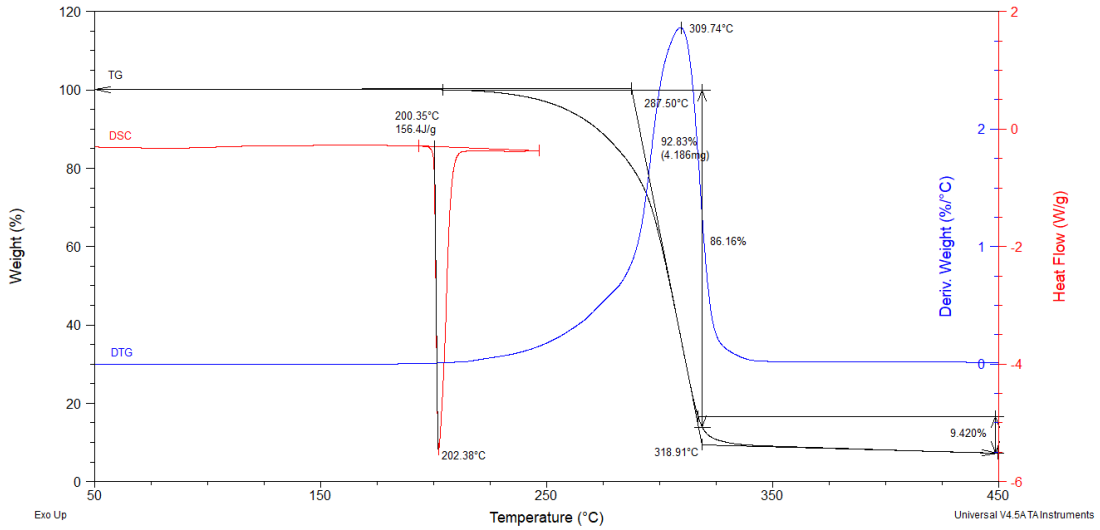
Figure 13. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of sulfamethazine obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



4.2.2 Trimethoprim (TMP)

In the TG/DTG curves (Figure 14), the TMP was thermally stable up to a temperature of 216.02°C.

Figure 14. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of trimethoprim obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



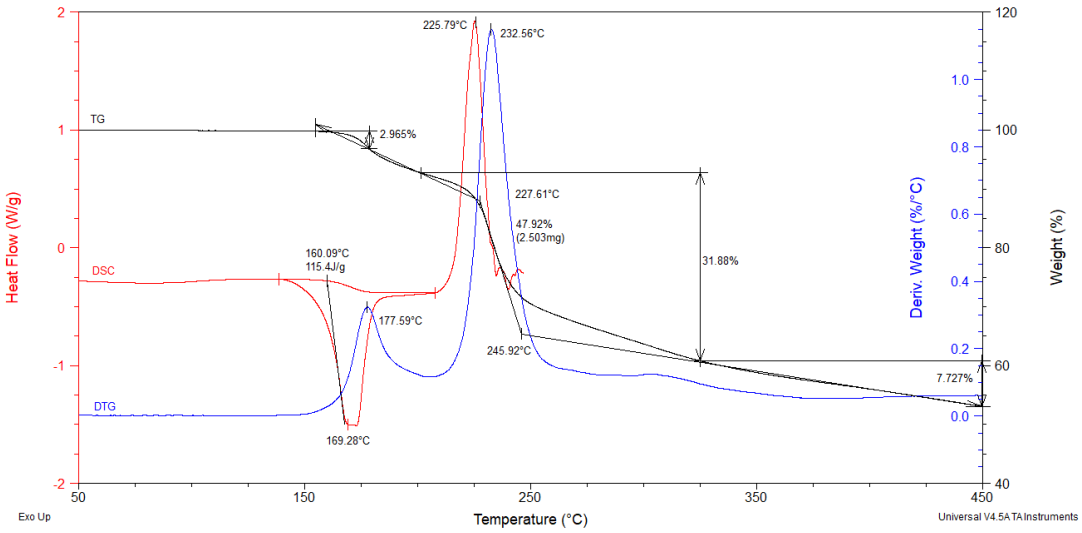
In the range from 287.50 to 318.91°C, mass loss was observed, with a variation of 92.83%. The total mass loss was approximately 94.86%, producing approximately 5.133% of residue. The DTG curve showed an exothermic peak at 309.74°C.

In the DSC curve, an endothermic peak was observed between 193.85°C and 213.13°C attributed to the melting of TMP. The calculated melting T*onset* was 202.38°C with an enthalpy of 156.4 J g^{-1} and 200.35°C, being in accordance with the melting range of 199°C to 203°C reported by Moffat, Osselton and Widdop (2011).

4.2.3 Doxycycline (DOXY)

As observed in the TG/DTG curves (Figure 15), DOXY remained thermally stable up to 158.02°C. Two more significant mass loss events occurred: between 170.57 and 183.78°C, with a mass variation of 7.246%, and at 227.61 to 245.92°C with a mass variation of 47.92%.

Figure 15. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of doxycycline obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



At the end of the heating process, the mass loss was 55.1%, leaving 44.90% of inorganic residue. The DTG curve showed two exothermic peaks at 177.59°C and 232.56°C.

In the DSC curve, an endothermic peak was observed between 139.02 and 207.84ºC, corresponding to the melting of the material. The calculated melting T*onset* was 169.28°C with a melting enthalpy of 115.4 J g^{-1} and 160.09°C, while Moffat, Osselton and Widdop (2011) mention that melting occurs at around 200°C with

decomposition, as observed in the product information report from Sigma-Aldrich (2019), which was around 201°C . The second peak observed in DSC is exothermic, characteristic of degradation at 225.79°C.

4.3 CHROMATOGRAPHIC CONDITIONS

Conducting system compliance analyses is essential for determining that the selected system is adequate to generate precise, accurate and reliable results during the analysis process (PASCHOAL et al., 2008).

To do so, we integrated together with the analytical procedures, the parameters: resolution, number of plates, asymmetry and repeatability, which indicate the selectivity of the system, the precision and the efficiency of the column (MUKHERJEE and BERA, 2012).

In parameter analysis, the following appropriate values are: repeatability: less than 1.0%, resolution: greater than 2.0, asymmetry: less than 2.0, platters: greater than 2000, and peak purity: greater than 950, and at least two of the previously mentioned criteria must be met to guarantee the system's conformity and that the data obtained have quality (RIBANI et al., 2004; MAIO et al., 2006).

The elution mode chosen was isocratic, as it provides a reduction in solvent consumption and does not require additional conditioning steps for the mobile phase. The mobile phase flow chosen was 0.5 mL min^{-1}, as it was observed that gradual reductions from 0.5 to 0.3 mL min^{-1} and 0.2 mL min^{-1} caused a broadening of the DOXY peak, in addition to increasing analysis time due to greater retention of drugs in the stationary phase. Flow rates greater than 0.5 mL min^{-1} were not tested in order to minimize the disposal of organic solvent residues.

In the analyses carried out with mobile phases composed of methanol and water without pH adjustment and with pH 3.0, adjusted with phosphoric acid, it was not possible to separate all drugs in any of the tested columns. The same happened with the mobile phases composed of acetonitrile, methanol and water at pH 3.0, adjusted with phosphoric acid.

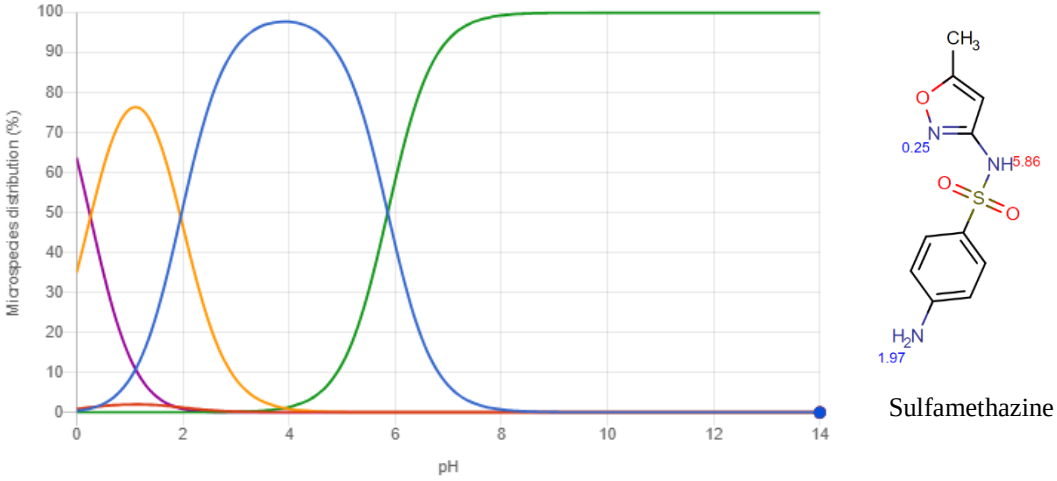
When carrying out tests with different proportions of acetonitrile and water, the mobile phase chosen as ideal for chromatographic analysis on the C8 Macherey-Nagel (MN)® column (4 mm x 125 mm, 5 µm) was: acetonitrile and water (45:55 v/v) with pH adjusted to 3.0 with phosphoric acid.

The choice of the pH of the mobile phase was based on information on the pKa and degree of ionization of the drugs at acidic and basic pH, in order to assist in the selection of the mobile phase and the chromatographic separation of the drugs, for less interaction with the reversed stationary phase, resulting in shorter retention time.

In reversed-phase chromatography, pH 2 to 8 is the recommended range, and it is essential to choose an appropriate pH so that the peak results are symmetrical and clear. At low pH, the mobile phase protonates the free silanols in the columns and reduces the peak tail (DESHMUKH et al., 2019).

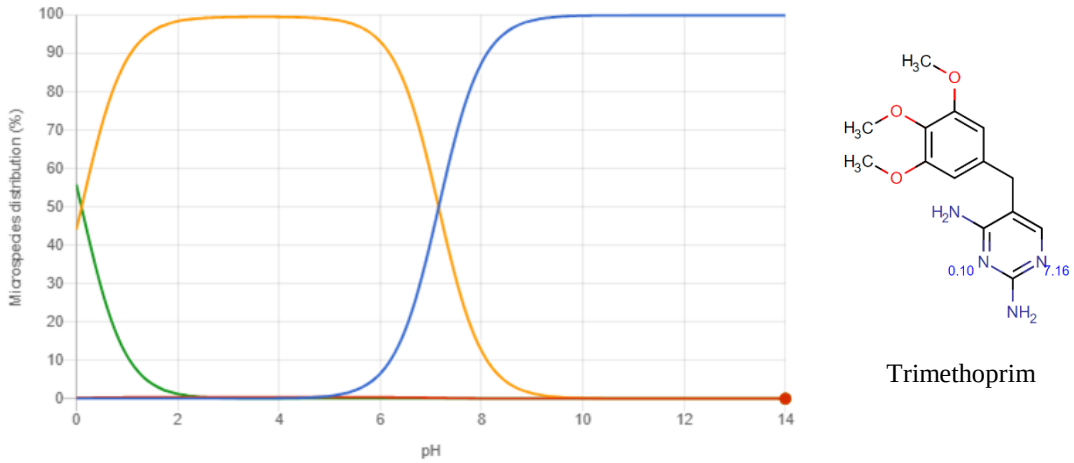
The pH value chosen for the mobile phase was 3.0, taking into account the pKa value of the drugs SMZ, TMP and DOXY and the presence of ionized species in an acidic environment, in order to favor solubility in the mobile phase and avoid adsorption in the stationary phase (Figures 16, 17 and 18).

Figure 16. Distribution of sulfamethazine microspecies (%) as a function of pH.



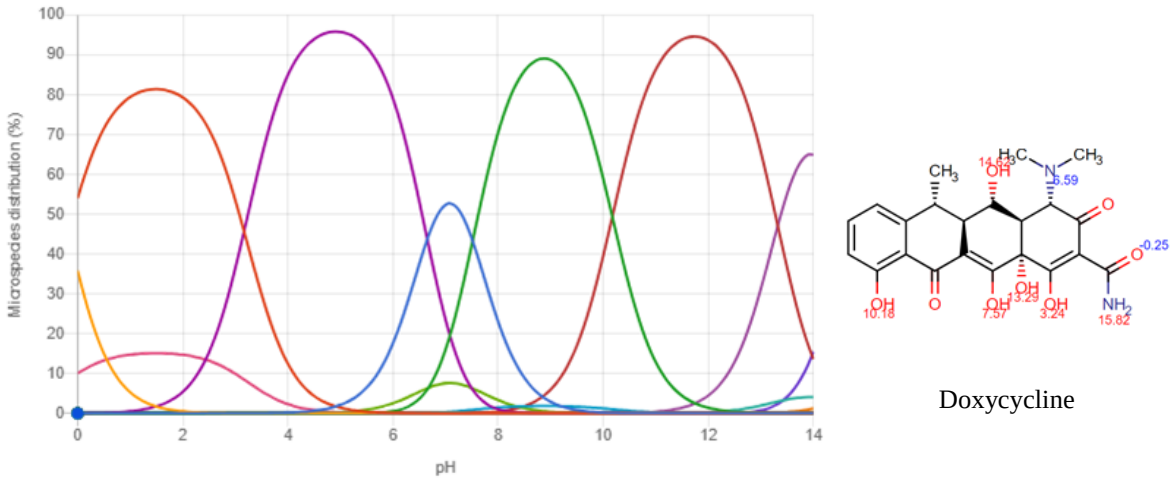
Source: Chemicalize, 2023.

Figure 17. Distribution of trimethoprim microspecies (%) as a function of pH.



Source: Chemicalize, 2023.

Figure 18. Distribution of doxycycline microspecies (%) as a function of pH.



Source: Chemicalize, 2023.

In the analyzes carried out with the C18 ThermoScientific®, C18 Zorbax Agilent®, C18 Kromasil® and C18 InertSustain GL Sciences® columns, it was not possible to obtain a good separation of the drugs in any of the proportions of mobile phases and flow rates tested.

The analyzes carried out with the C8 column from the manufacturer GL Sciences® resulted in a good separation of the drugs, however the drugs TMP and DOXY presented the number of theoretical plates and asymmetry of the chromatographic peaks below that recommended by the literature. Furthermore, the chromatographic peak of the drug SMZ also presented a resolution below the minimum established in the literature.

Therefore, among the columns analyzed, only the C8 column from the manufacturer Macherey-Nagel (MN)® presented adequate results in the parameters of resolution, asymmetry, thermal plates and purity for the drugs SMZ, TMP and DOXY.

After conducting tests with several chromatographic conditions: columns, solvent mixtures, flow and pH of the mobile phase, the method for the simultaneous determination of SMZ, TMP and DOXY was developed with the conditions that produced the best results in the parameters: resolution, asymmetry, plates and peak purity, ensuring system compliance and data quality, indicating system selectivity, column accuracy and efficiency (MUKHERJEE and BERA, 2012).

Table 8 shows the chromatographic conditions selected for the development and validation of the analytical method: Macherey-Nagel (MN)® C8 analytical column (4 mm x 125 mm, 5 µm), flow rate of 0.5 mL min^{-1} and mobile phase composed of acetonitrile:water (45:55 v/v) with pH adjusted to 3.0.

After choosing the column, mobile phase and flow for the analysis of the drugs and development of the analytical method, the wavelengths for the quantification of the drugs were selected. Spectral scans were performed with the aid of the diode array detector (DAD) in the range 200-400 nm. The wavelengths selected for SMZ, TMP and DOXY quantification were 268 nm, 270 nm and 350 nm, respectively, corresponding to the regions of maximum UV absorption of each drug.

Table 8. Parameters evaluated in the analysis of conformity of the chromatographic system with the Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm).

Drug	Evaluated Parameters[a,b]				
	Retention time (min)	**Resolution**	**Asymmetry**	**Theoretical Plates**	**Purity**
Sulfamethazine	2.40 ± 0,12	7.18 ± 0.17	1.14 ± 0.38	13.373 ± 0.19	999 ± 0.26
Trimethoprim	2.87 ± 0.15	4.70 ± 0.13	1.26 ± 0.25	12.038 ± 0.28	998 ± 0.14
Doxycycline	3.17 ± 0.13	-	1.45 ± 0.51	5.869 ± 0.33	997 ± 0.58

a Conditions employed: mobile phase composed of acetonitrile:water (45:55 v/v) pH 3.0 and flow rate of 0.5 mL min^{-1}. b Mean ± coefficient of variation of 6 determinations.

The analysis of the purity of the drug peaks was carried out by evaluating the similarity of the UV spectra extracted from the chromatographic peaks, obtained by the diode array detector (DAD) of the chromatograph.

In Figure 19, it is possible to observe the superimposed drug spectra, indicating that the chromatographic peaks of sulfamethazine, trimethoprim and doxycycline are pure, therefore, there is no co-elution of interferents (purity > 997).

In Figure 20, the ungrouped UV spectra of sulfamethazine (A), trimethoprim (B) and doxycycline (C) are presented, showing the absence of co-elution of interferents and spectral homogeneity in the wavelength of the drugs analyzed. Therefore, no significant differences were found between the extracted spectra.

Figure 19. Analysis of the purity of sulfamethazine (A), trimethoprim (B) and doxycycline (C) peaks, obtained by the diode array detector, by evaluating the similarity of the UV spectra.

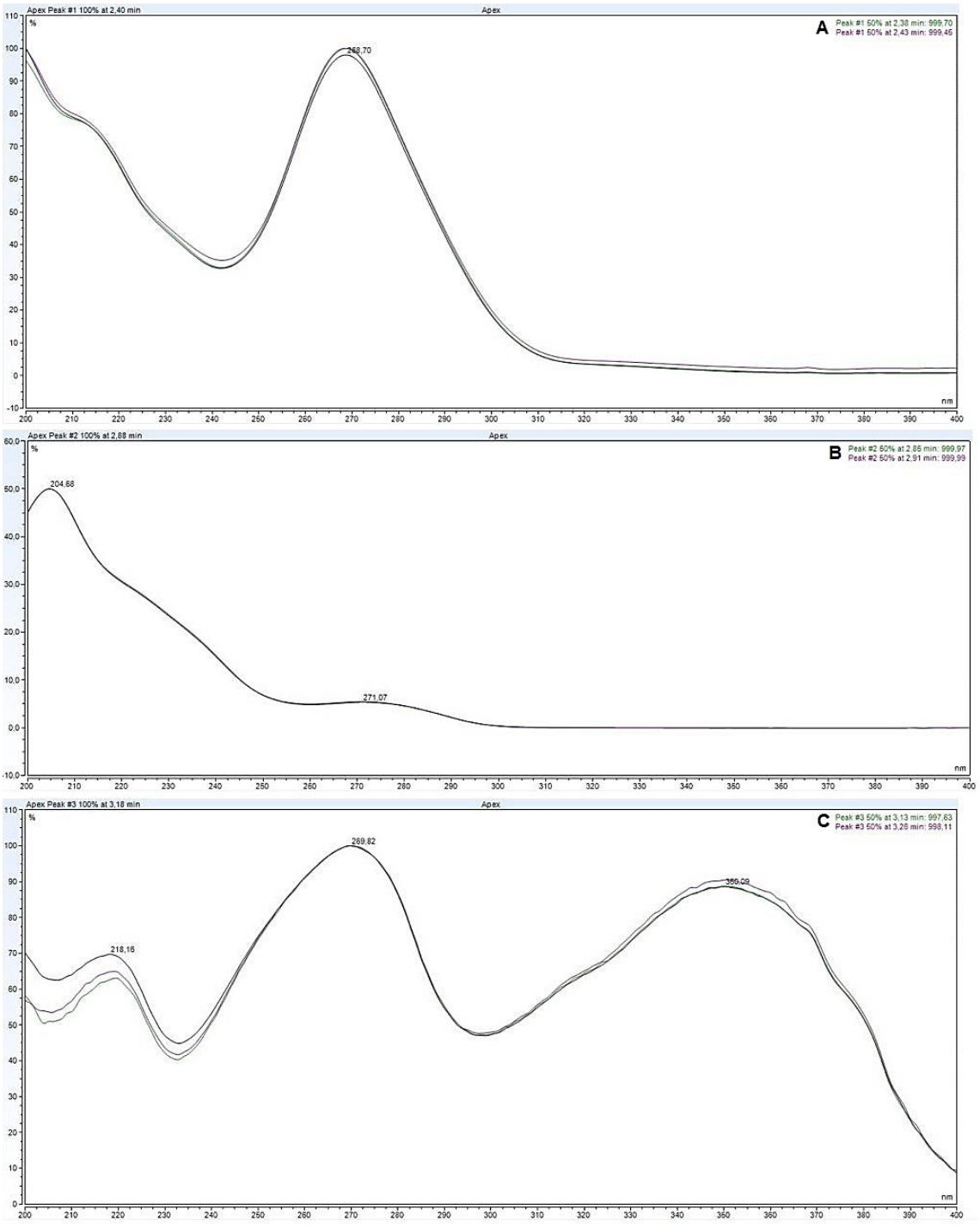


Figure 20. Analysis of the purity of sulfamethazine (A), trimethoprim (B) and doxycycline (C) peaks, by evaluating the similarity of the ungrouped UV spectra, obtained by the diode array detector.

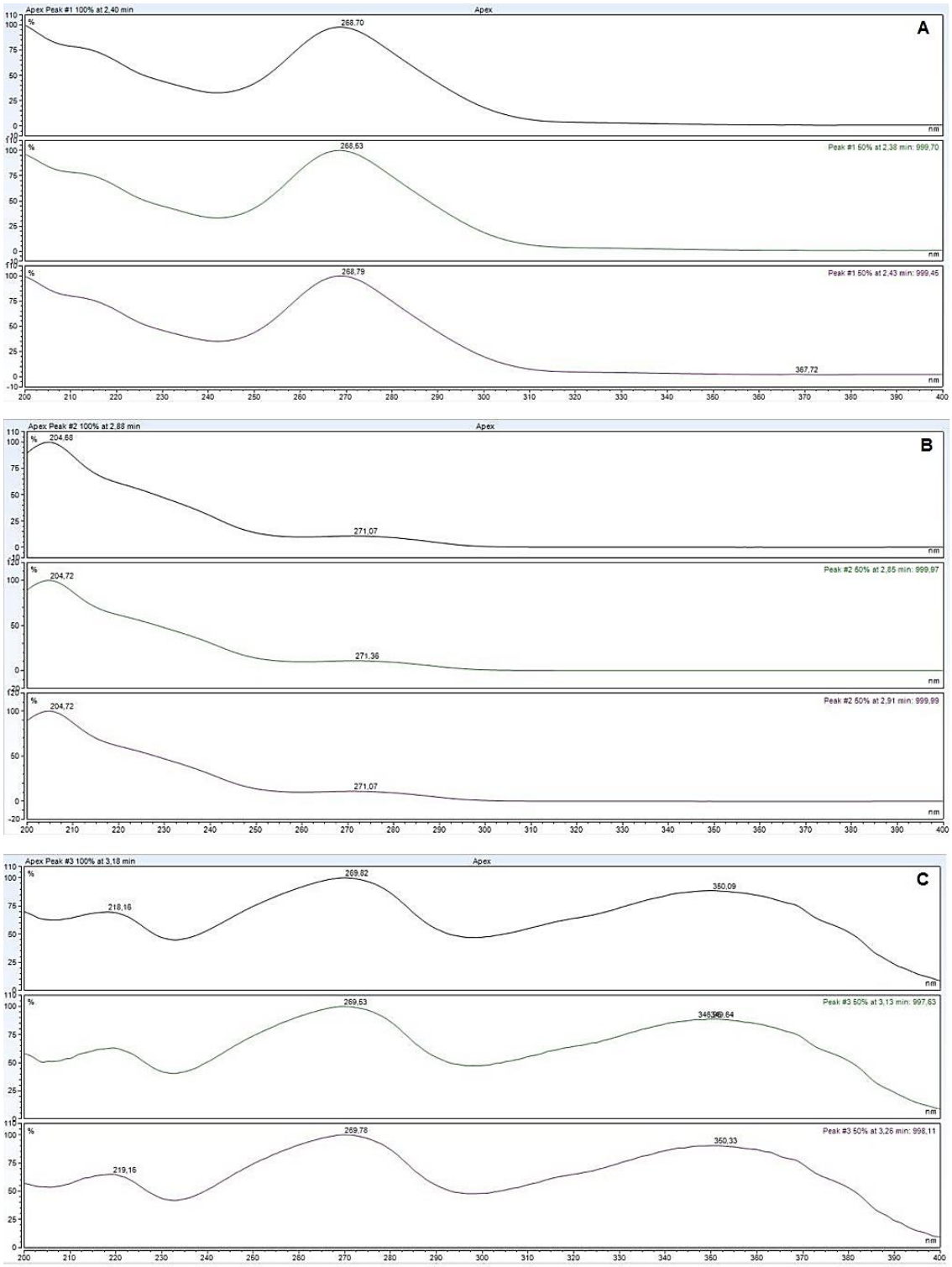
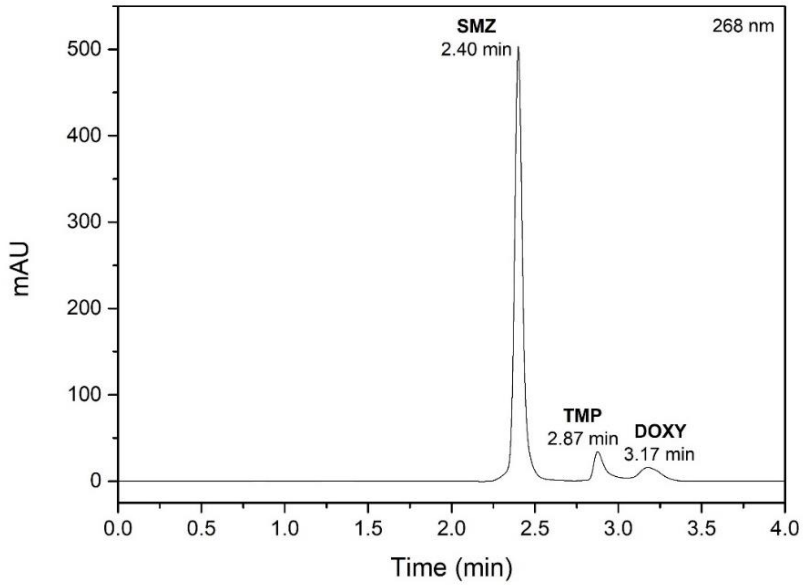


Figure 21 shows the chromatogram of the chromatographic separation of the solution of the drugs SMZ, TMP and DOXY, under the developed chromatographic conditions. In this figure, it is possible to verify the good chromatographic separation of

the three drugs, in a short time of analysis (4 minutes), indicating that the method is suitable for validation and simultaneous determination of drugs in pharmaceutical formulations for animal use.

Figure 21. Chromatogram of the sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) solution prepared for system compliance analysis and analyzed under the chromatographic conditions: coluna Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm), flow rate of 0.5 mL min^{-1} and mobile phase composed of acetonitrile:water (45:55 v/v) with pH adjusted to 3.0.



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4.3.1 Validation of the Analytical Methodology

4.3.1.1 Linearity, Limits of Detection (LD) and Quantification (LQ)

Table 9 describes the equations of the line, the correlation coefficients and LQ and LD obtained from the analytical curves of the drugs under study.

Table 9. Results obtained from the calibration curves performed in the linearity test, limits of detection and quantification.

Drug	Interval (µg mL^{-1})	Line equation	R	LD (µg mL^{-1})	LQ (µg mL^{-1})
Sulfamethazine	5.0 – 35.0	y = 2.7693x + 0.2697	0.9999	0.114	0.345
Trimethoprim	1.0 – 7.0	y = 1.1328x + 0.0152	0.9998	0.034	0.102
Doxycycline	7.0 – 13.0	y = 0.2982x + 0.0039	0.9999	0.072	0.219

R: Correlation coefficient; LD: limit of detection; LQ: Limit of quantification.

The analytical curves were linear, as they presented correlation coefficients close to 1.0 (r> 0.9997) in the concentration range used for drug analysis, demonstrating that

the results obtained were directly proportional to the concentration of the analyte in the sample, within the specified interval.

The LD and LQ values indicated that the proposed method, since it can determine small concentrations of the analyzed drugs, demonstrating to be sensitive for simultaneous determination of drugs in pharmaceutical formulations.

Table 10 describes the results of the statistical analysis performed by the ANOVA test, in which it was possible to observe that the linear regression was significant for the drugs analyzed (Fcalculated>Critical), therefore, the concentration of the analytes is correlated with the signal generated by the equipment (peak area).

Table 10. Analysis of variance (ANOVA) of the linear regression of the drugs sulfamethazine, trimethoprim and doxycycline.

Drugs	Source of variation	DF	SQ	MS	F calculated	F critical
Sulfamethazine	Regression	1	5368.24097	5368.24097	92711.52775	$7.25131 .10^{-13}$
	Residue	5	0.28951	0.0579		
	Total	6	5368.53049			
Trimethoprim	Regression	1	35.93007	35.93007	42847.73774	$4.99316 .10^{-11}$
	Residue	5	0.00419	$8.38552 .10^{-4}$		
	Total	6	35.93427			
Doxycycline	Regression	1	2.49047	2.49047	56548.65713	$2.49555 .10^{-11}$
	Residue	5	$2.20206 .10^{-4}$	$4.40411 .10^{-5}$		
	Total	6	2.49069			

DF: degree of freedom; SQ: sum of squares; MS: mean squares.

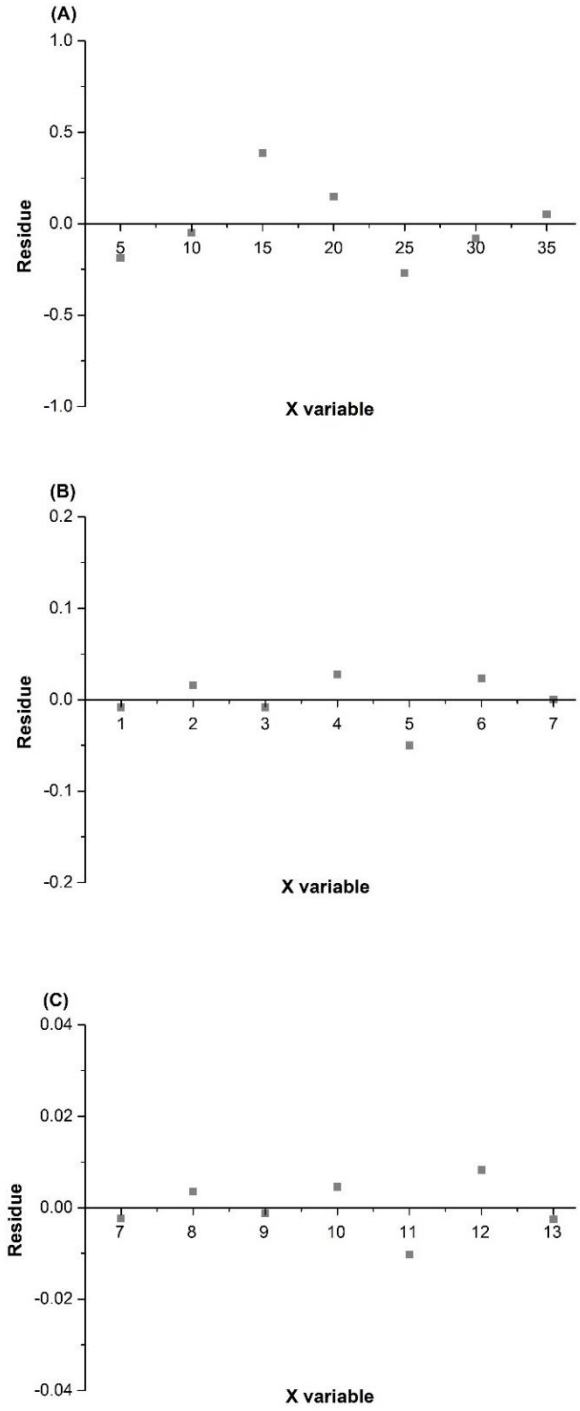
In addition to the analytical curves and the ANOVA test, linearity can also be verified through the regression analysis of residues.

The residual is considered the difference between the values observed in the measurements of each point of the calibration curve, therefore, from the analysis of residuals it is possible to see if there are divergent data and if the variance of the data is homogeneous or not (AMADOR et al., 2011).

The linearity of the method was also analyzed through the regression analysis of the residues for the evaluation of normality (BRASIL, 2017b).

Figure 22 shows the graphs showing the distribution of residues as a function of the analytical response of the analyzed drugs. It was verified that the residues are homogeneously distributed, with constant variance, indicating the homoscedasticity of the variances and close to the zero axis, with no values outside the expected range.

Figure 22. Analysis of residues obtained from the calibration curves of (A) SMZ (sulfamethazine), (B) TMP (trimethoprim) and (C) DOXY (doxyxycline).



4.3.1.2 Precision

Table 11 describes the results obtained with the precision test of the drugs analyzed using the developed method. The values obtained in the determinations made with the drugs SMZ, TMP and DOXY were expressed in terms of relative standard deviations

(RSD), observing that the values were lower than the maximum recommended limit of 5.0% (ICH, 2005), confirming the accuracy of the developed analytical method.

Table 11. Results obtained in the precision parameter of the analytical method.

			Content ± RSD (%)		
	Day	**Analyst**	**SMZ**	**TMP**	**DOXY**
Precision intermediate	1	1	101.18 ± 0.12	99.92 ± 0.21	100.81 ± 0.19
		2	101.21 ± 0.16	99.96 ± 0.28	100.82 ± 0.24
	2	1	101.26 ± 0.30	99.89 ± 0.29	100.85 ± 0.20
		2	101.29 ± 0.27	99.87 ± 0.28	100.89 ± 0.29
	3	1	101.23 ± 0.39	100.03 ± 0.32	100.82 ± 0.17
		2	101.20 ± 0.31	100.01 ± 0.23	100.86 ± 0.21
		Mean	101.23 ± 0.26	99.94 ± 0.27	100.84 ± 0.22
Repeatability			101.19 ± 0.18	99.97 ± 0.21	100.85 ± 0.16

RSD: relative standard deviation; SMZ: sulfamethazine; TMP: trimethoprim; DOXY: doxycycline.

4.3.1.3 Robustness

The effects observed in the robustness analysis are shown in Figure 23. The critical values ($\sqrt{2}S$) in relation to the levels of SMZ, TMP and DOXY for the higher levels were, respectively, 1.09, 1.20 and 1.24 (Figure 23A), and for the lower levels, 1.26, 1.22 and 1.15 (Figure 23B).

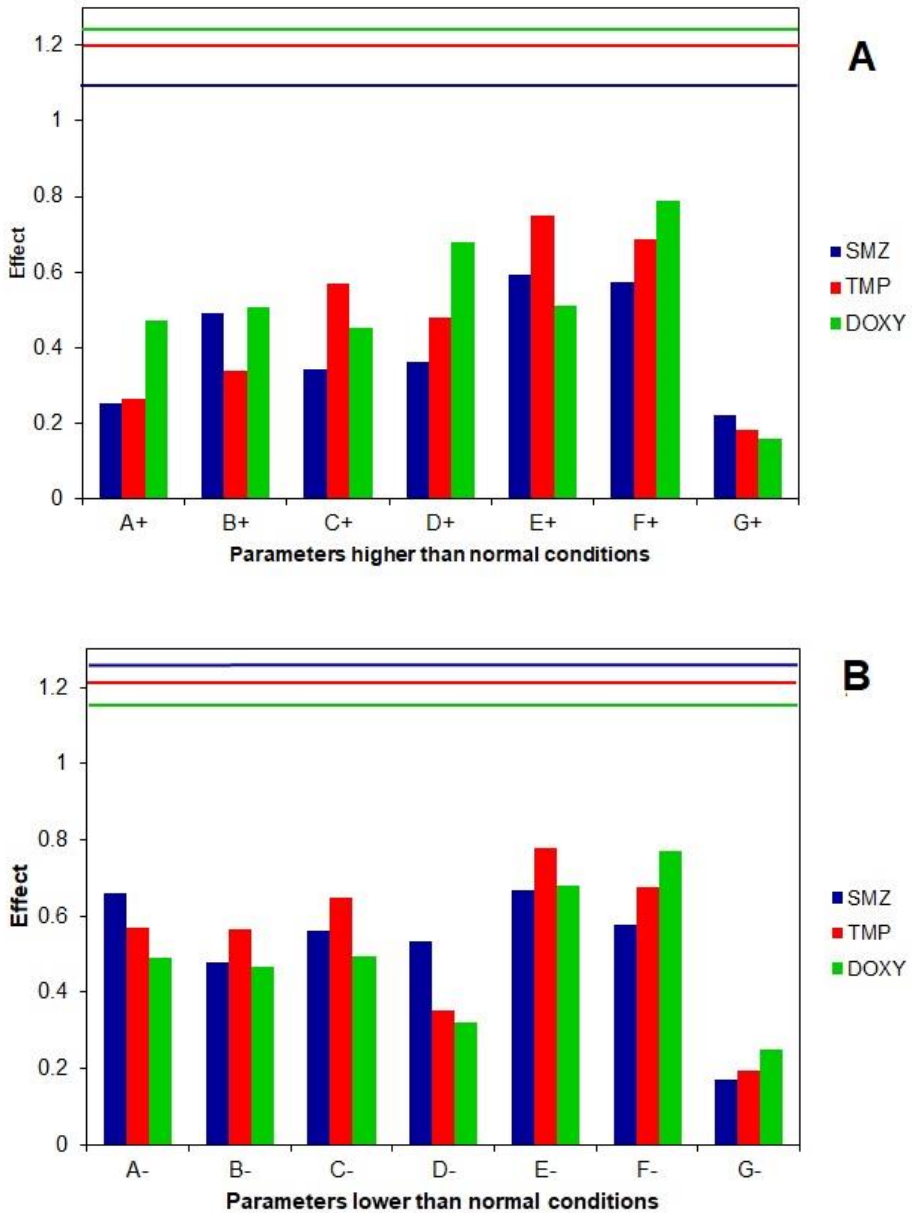
Of the modified conditions, none significantly influenced the contents of the drugs, not exceeding the critical values, as well as in the retention times, asymmetry, resolution and number of plate.

However, under conditions above normal in the wavelength factor, DOXY showed the greatest variation in relation to the other factors, while SMZ and TMP had the greatest variation in the pH of the mobile phase.

Among the variations carried out under conditions below normal, the pH factor of the mobile phase of SMZ and DOXY, was the one that showed the greatest effect in relation to the other factors. The TMP had a greater effect on the wavelength factor in relation to the variation performed on other factors.

In view of this, the proposed method proved to be robust for the simultaneous determination of SMZ, TMP and DOXY, since the effects observed on the factors selected in the analysis were smaller than the critical values found in the inferior and superior conditions, suggesting that the method is capable of withstand small variations.

Figure 23. Effects and critical values observed in the robustness assay of sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) by the Plackett-Burman test: (A) higher than normal conditions (+1) (B) less than normal conditions (-1). The blue, red and green lines represent the critical values for SMZ, TMP and DOXY drugs, respectively. Letters from A to G represent the selected factors: A: sonification time; B: mobile phase flow; C: acetonitrile supplier; D: mobile phase proportion; E: mobile phase pH; F: wavelength; G: laboratory temperature.



4.3.1.4 Accuracy

The accuracy of the method was determined through the recovery tests. The values obtained for the recovery of the amount of standard solution of each drug are shown in Table 12.

Table 12. Accuracy test of the drugs sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY), analyzed by the proposed chromatographic method.

Drugs	Theoretical concentration added (μg mL^{-1})	Experimental theoretical concentration (μg mL^{-1})	Recovery (%)	
			Result (%)	Mean ± RSD (%)
SMZ	25.00	24.97	99.00	99.36 ± 0.31
	30.00	29.99	99.46	
	35.00	34.99	99.60	
TMP	4.00	3.99	99.39	99.23 ± 0.16
	5.00	4.97	99.06	
	6.00	5.97	99.25	
DOXY	10.00	9.98	99.44	99.42 ± 0.15
	12.00	11.96	99.26	
	14.00	13.97	99.56	

RSD: Relative standard deviation

The recovered amounts of SMZ, TMP and DOXY were within acceptable limits of 98-102% for all three concentration levels analyzed and the RSD of the average of these levels was below 2.0% (AOAC, 2005). The mean recovery percentages of SMZ, TMP and DOXY were 99.36%, 99.23% and 99.42%, respectively. Therefore, the developed method has adequate accuracy for simultaneous determination of SMZ, TMP and DOXY.

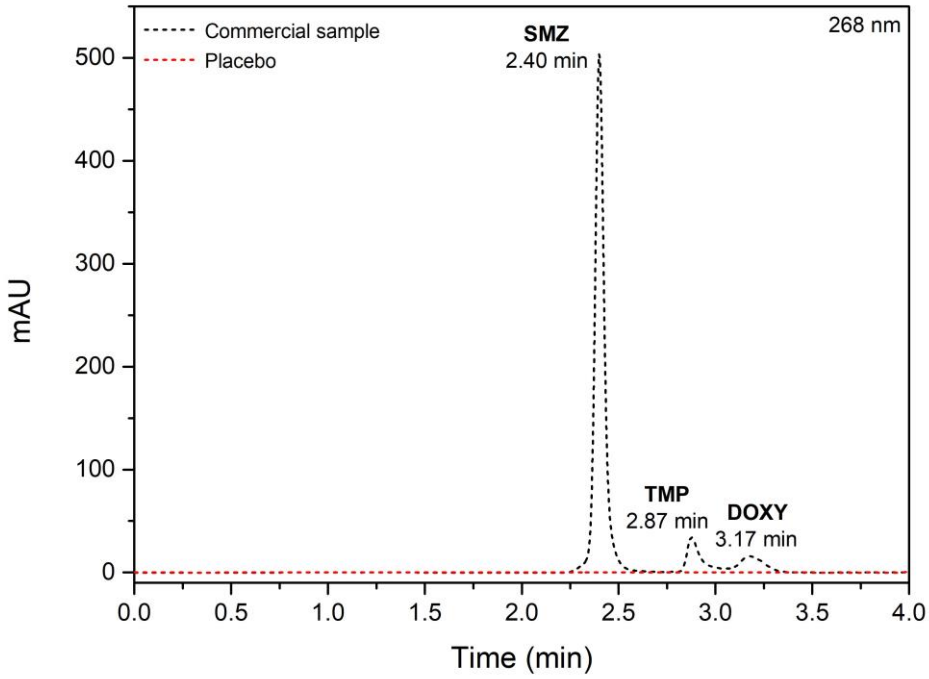
4.3.1.5 Selectivity

Figure 24 shows the chromatogram of the adjuvant solution (placebo) superimposed on the sample chromatogram commercial.

The adjuvants used for the selectivity analysis did not interfere with the simultaneous determination of SMZ, TMP and DOXY, since they did not show any peak eluting at the same retention time of the analyzed drugs and the peak purity was greater than 950, indicating that there was no coelution with other substances.

Therefore, this result demonstrates the selectivity of the method developed for the simultaneous analysis of SMZ, TMP and DOXY in pharmaceutical formulations for animal use.

Figure 24. Chromatogram of the adjuvant solution superimposed on the chromatogram of the commercial sample solution, analyzed using the proposed chromatographic method. SMZ: sulfamethazine; TMP: trimethoprim; DOXY: doxycycline.



Source: Own author using Origin Pro 9.0

4.4 METHOD APPLICABILITY

The analytical method developed and validated by HPLC-DAD proved to be suitable for simultaneous determination, qualification and quantification of the association of SMZ, TMP and DOXY in pharmaceutical formulations for animal use. For the commercial formulation Doxigram ST® the levels found were 101.59 ± 0.13% SMZ, 99.82 ± 0.18 TMP and 100.88 ± 0.21 DOXY, being within the limits recommended by official compendia, of at least 90% and a maximum of 110%.

In Table 13, the drug levels in the analysis of the commercial sample using the proposed method are described.

These results demonstrated the applicability of the HPLC-DAD method developed and validated for simultaneous determination, qualification and quantification of the association of SMZ, TMP and DOXY in pharmaceutical formulations for animal use.

Table 13. Analysis of the commercial sample using the chromatographic method: Macherey-Nagel (MN)® C8 analytical column (4 mm x 125 mm, 5 µm), mobile phase composed of acetonitrile:water (45:55 v/v) pH 3.0 and flow rate of 0.5 mL min^{-1}.

Drug	Content found ± RSD (%)[a]		
	SMZ	TMP	DOXY
Commercial sample	101.87 ± 0.09	99.81 ± 0.19	100.86 ± 0.26
	101.41 ± 0.17	99.86 ± 0.15	100.98 ± 0.19
	101.49 ± 0.14	99.78 ± 0.21	100.81 ± 0.17

a mean of 3 determinations ± relative standard deviation (RSD); SMZ: sulfamethazine, TMP: trimethoprim, DOXY: doxycycline.

4.5 FORCED DEGRADATION STUDY

In the forced degradation study, the drug degradation percentages were determined, under the conditions selected for the degradation study of the association SMZ, TMP and DOXY. In table 14, it is possible to observe that the drugs had different percentages of degradation, and not all conditions allowed the degradation within the recommended limit (10 to 30%) (WHO, 2009).

Table 14. Percentage of drug degradation, under the conditions selected to carry out the forced degradation study of sulfamethazine, trimethoprim and doxycycline.

Condition of degradation	Stress condition	Exposure time	Degradation (%)		
			SMZ	TMP	DOXY
Alkaline hydrolysis	NaOH 0.01 M	4 hours	27.25	15.02	18.81
Acid hydrolysis	HCl 0.1 M	8 hours	11.92	9.85	13.69
Neutral hydrolysis	H_2O	30 minutes	18.11	14.45	11.84
Oxidative hydrolysis	H_2O_2 0.03%	30 minutes	27.85	20.99	26.69
Photolysis	Light UV/Vis[a]	16 hours	26.99	13.28	29.35

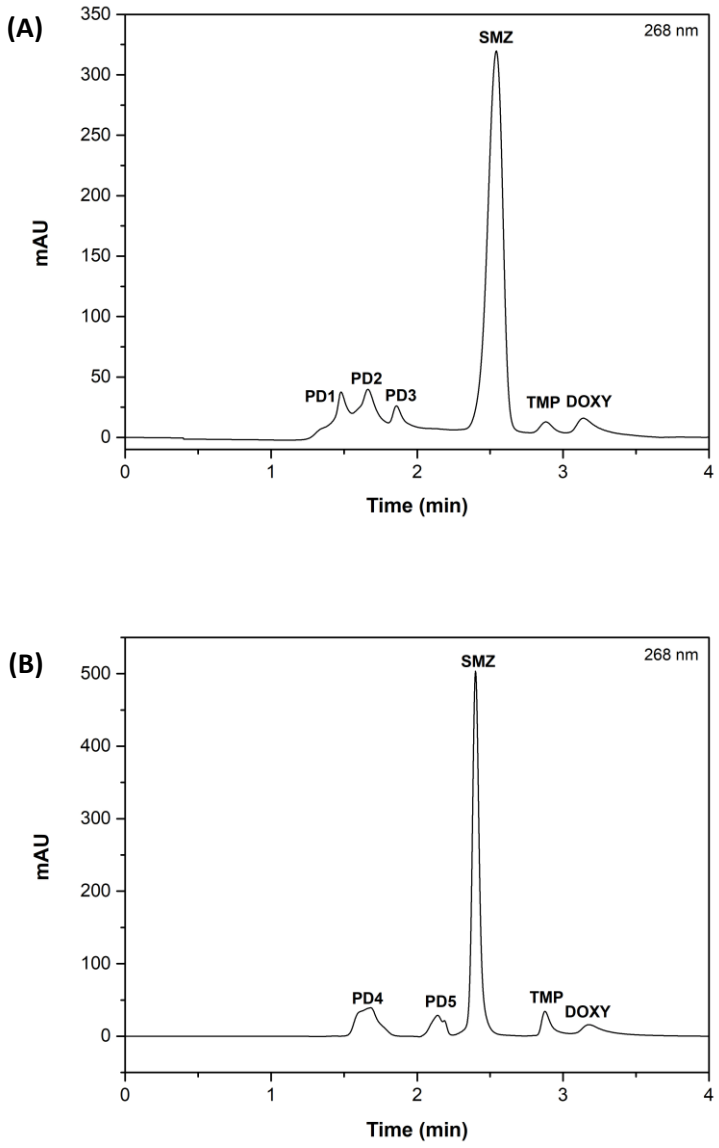
Room temperature (24 ± 2°C); [a]lamps UV (254-365 nm); SMZ: sulfamethazine; TMP: trimethoprim; DOXY: doxycycline.

Forced degradation studies help to obtain information about the intrinsic stability of drugs, facilitating the anticipation of future stability problems of the finished product and better choice of pharmaceutical form, packaging, transport and storage conditions, in order to avoid the formation of degradation products (SINGH et al., 2013; KOGAWA and SALGADO, 2016).

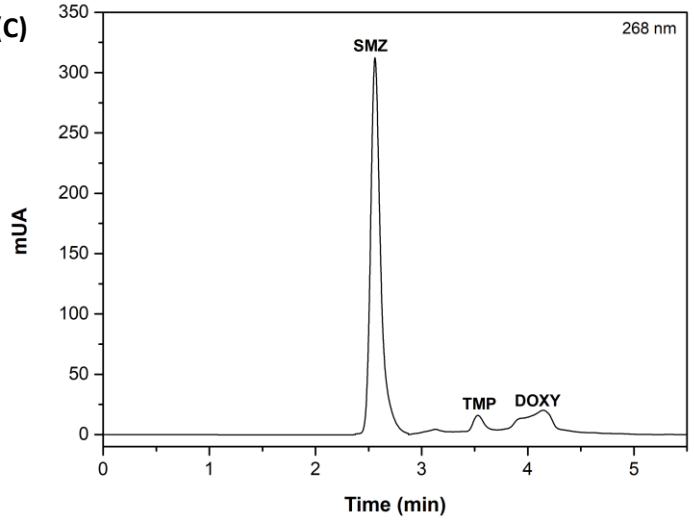
The chromatograms of the association of SMZ, TMP and DOXY, obtained from the method developed and validated in the forced degradation study, are illustrated in Figure 25.

No major changes were observed between drug retention times during stability studies, however, the drug content decreased under the different conditions of forced degradation analyzed.

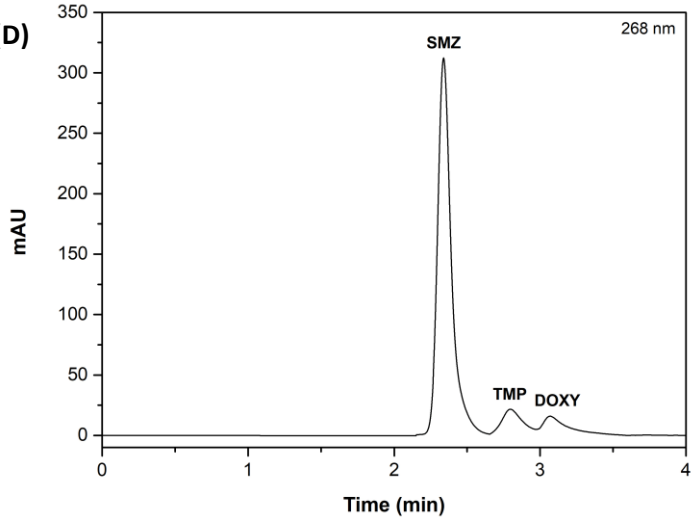
Figure 25. Chromatrograms of the combination of sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY); (A) basic hydrolysis (0.01 M NaOH, 4 h), (B) acid hydrolysis (0.1 M HCl, 8 h), (C) oxidative hydrolysis (0.03% H_2O_2, 30 min), (D) neutral hydrolysis (H_2O, 30 min); (E) Photolysis (UV/VIS light, 16 h). Chromatographic conditions: Macherey-Nagel (MN) C8 analytical column (4 mm x 125 mm, 5 µm), flow rate of 0.5 mL min^{-1} and mobile phase composed of acetonitrile:water (45:55 v/v) with pH adjusted to 3.0.



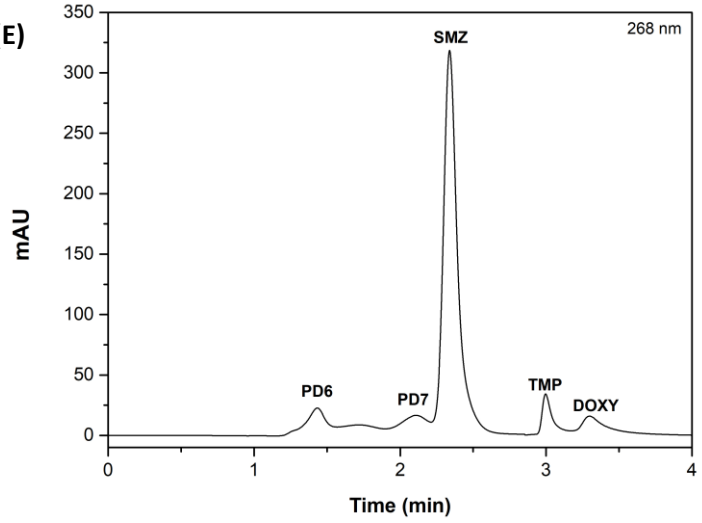
(C)


268 nm
SMZ
TMP
DOXY
mUA
Time (min)

(D)


268 nm
SMZ
TMP
DOXY
mAU
Time (min)

(E)


268 nm
SMZ
PD6
PD7
TMP
DOXY
mAU
Time (min)

The purity of the chromatographic peaks of the drugs was greater than 980 for all degradation conditions, which indicates that there was no co-elution of degradation products, indicating the selectivity of the method (STAHL, 2003; MAIO et al., 2006). Furthermore, the presence of seven degradation products was observed, three in basic hydrolysis, two in acid hydrolysis and two in oxidative hydrolysis.

In alkaline hydrolysis (Figure 22A), drug degradation was above the minimum limit of 10%, SMZ, TMP and DOXY were degraded by 27.25%, 15.02% and 18.81%, respectively.

Under acid hydrolysis (Figure 22B), SMZ, TMP and DOXY degraded 11.92%, 9.85% and 13.69%, respectively. In this condition, only the TMP presented degradation below the minimum limit of 10%.

In oxidative hydrolysis (Figure 22C), SMZ, TMP and DOXY degraded 27.85%, 20.99% and 26.69%, close to the maximum recommended limit of 30%. Furthermore, a change in the shape presented by the DOXY peak was observed.

In neutral hydrolysis (Figure 22D), the percentage of drug degradation was 18.11% SMZ, 14.45% TMP and 11.84% DOXY, and no degradation products were observed.

In the photolysis analysis (Figure 22E), the presence of two manipulation products was observed, SMZ presented a manipulation percentage of 26.99%, TMP 13.28% and DOXY 29.35%, close to the maximum limit (30%), the which can be significantly attributed to photodegradation and increased photosensitivity of this drug. According to Halling-Sorensen and Sengel (2002), drugs from the tetracycline class may present degradation products by direct photolysis, as they photodecompose easily.

Therefore, the method developed and validated by HPLC-DAD can be considered indicative of stability for simultaneous determination of the association of SMZ, TMP and DOXY, as it allows an efficient separation of drugs from degradation products in the analyzed sample.

4.6 ACCELERATED STABILITY

The quality of a product requires that the stability of the product be evaluated, from the selection of the substance to the registration of the product for commercialization in the market (CHA et al., 2011).

The stability study must be carried out at specific times to establish the stability profile of the substance, with at least three periods in accelerated stability (0, 3 and 6 months) (POTNURI et al., 2018).

In the organoleptic evaluation of the commercial sample, no alterations in color and odor were observed, that is, the sample maintained the same odor and beige color from the beginning to the end of the stability study (Figure 26). However, the preservation of the color and odor of the sample is not indicative of the absence of alterations in the therapeutic content and efficacy.

Figure 26. Evaluation of the organoleptic characteristics of the commercial sample before (time zero), during (3 months) and after the end of the accelerated stability study (6 months).

Carrying out stability studies is essential to guarantee the quality, efficacy and safety of medicines, as through these it is possible to verify whether the quality of medicines is modified in the presence of factors such as light, humidity and temperature, and whether there is an occurrence of degradation (GONZÁLEZ-GONZÁLEZ et al., 2022).

In the analysis of the pH of the samples at time zero, during (3 months) and after the end (6 months) of the accelerated stability study, it was observed that there were no great variations in the pH values of the samples (Table 15). The contents of SMZ, TMP and DOXY drugs, obtained at different times analyzed during the accelerated stability study of the sample are shown in Table 16.

Table 15. Commercial sample pH analysis at time zero, 3 months and 6 months in the accelerated stability study.

	Accelerated Stability	
	Acclimatization time (months)	**pH[a] ± SD**
Commercial sample	0	5.58 ± 0.08
	3	5.61 ± 0.05
	6	5.55 ± 0.07

[a] mean of 3 determinations ± standard deviation (SD)

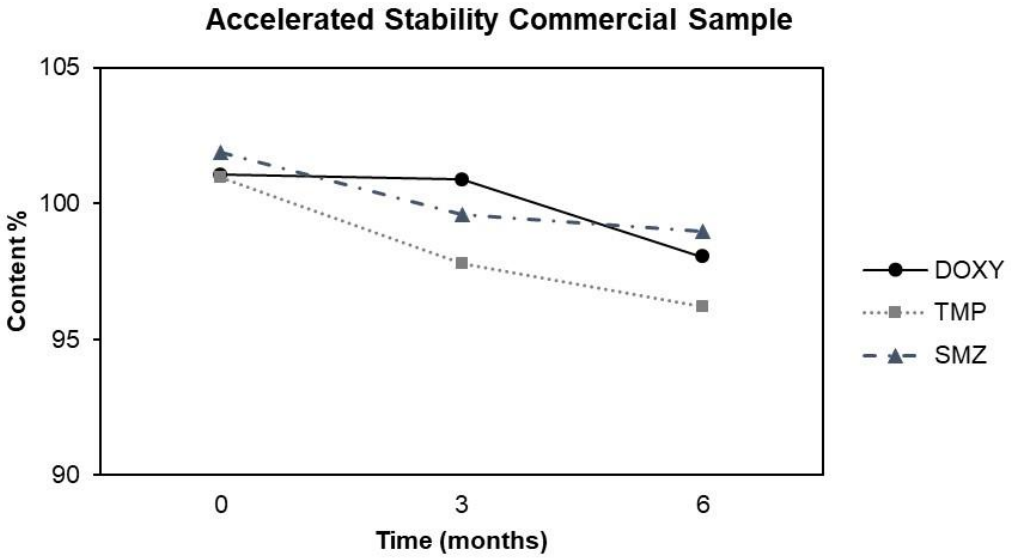
Table 16. Drugs content in the commercial sample submitted to the accelerated stability study.

Acclimatization time (months)	Content ± RSD (%)[a]		
	SMZ	TMP	DOXY
0	101.89 ± 0.08	100.94 ± 0.06	101.06 ± 0.12
3	99.58 ± 0.14	97.79 ± 0.08	100.87 ± 0.26
6	98.96 ± 0.20	96.18 ± 0.16	98.04 ± 0.14

[a]mean of 3 determinations ± relative standard deviation (RSD); SMZ: sulfamethazine, TMP: trimethoprim, DOXY: doxycycline.

The determination of drug contents was performed using the HPLC analytical method previously developed and validated. Figure 27 shows the decay profiles of the drug content present in the commercial sample, submitted to the accelerated stability test. The highest percentage of content decay was for TMP (4.76%), followed by DOXY (3.02%) and SMZ (2.93%).

Figure 27. Decay profiles of the drugs (SMZ: sulfamethazine, TMP: trimethoprim, DOXY: doxycycline) content present in the commercial sample, submitted to the accelerated stability test.



In view of the results obtained in this stability test, it is emphasized that these are fundamental to ensure the quality and efficacy of medicines, to avoid therapeutic inefficiency and toxic degradation products for patients and animals in commercialized medicines (WATERMAN and ADAMI, 2005; MORENO and SALGADO, 2012).

Therefore, accelerated stability studies are essential for evaluating chemical and physical effects in conditions outside those established on the product packaging.

4.7 ASSESSMENT OF DRUG-DRUG COMPATIBILITY

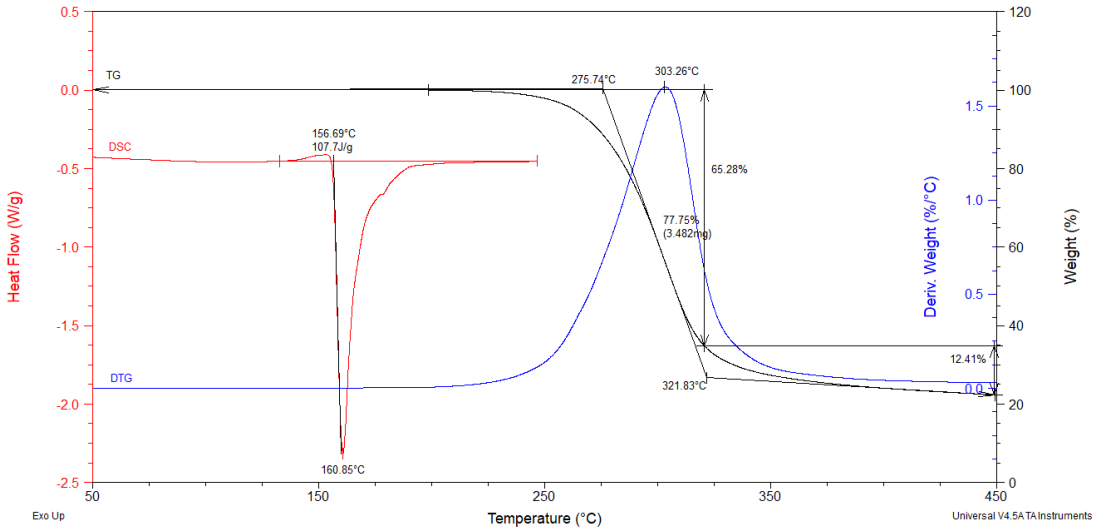
4.7.1 Binary Mixture of Sulfamethazine (SMZ) and Trimethoprim (TMP) (1:1 m/m)

In the TG/DTG curves (Figure 28), the mixture of SMZ and TMP remained thermally stable up to 204.44°C. Only one mass loss event was observed, between 275.74°C and 321.83°C, with a mass variation of 77.75%. At the end of the heating process, the mass loss was 81.40%, leaving 18.6% of inorganic residue. The DTG curve showed only one exothermic peak at 303.26°C.

In the DSC curve, an endothermic peak was observed between 133.35ºC and 208.22ºC, corresponding to the melting of the mixture, with a calculated melt T*onset* of 160.95°C with a melting enthalpy of 107.7 J g^{-1} and 156.69°C.

At this peak, it was not possible to identify whether the fusion was from SMZ or TMP, which may be suggestive of an interaction between the drugs due to heating, resulting in the probable formation of a eutectic mixture.

Figure 28. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the binary mixture of sulfamethazine and trimethoprim (1:1 m/m), obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



4.7.2 Binary Mixture of Sulfamethazine (SMZ) and Doxycycline (DOXY) (1:1 m/m)

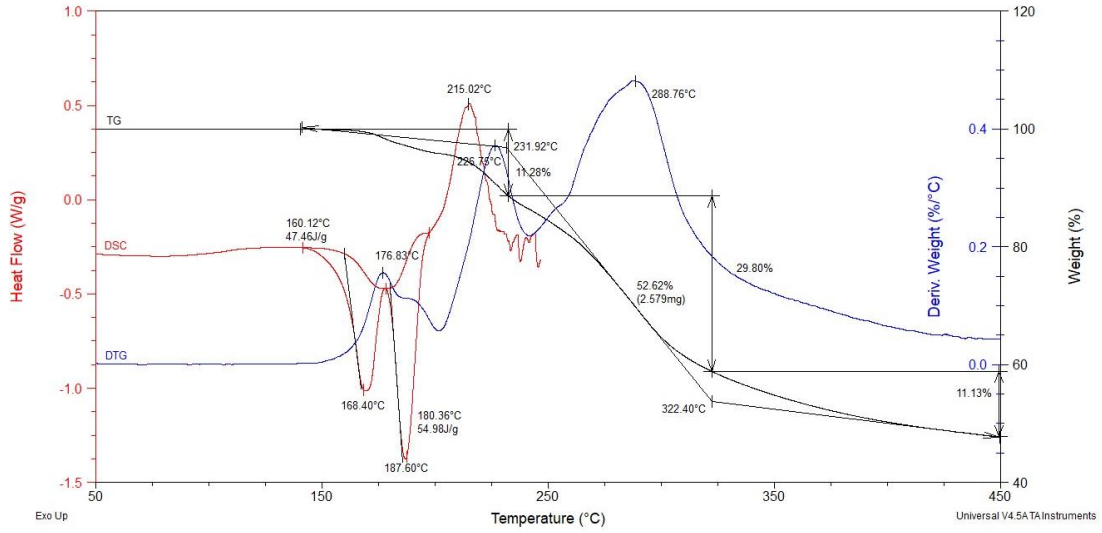
The TG/DTG curves of the mixture of SMZ and DOXY (Figure 29) demonstrated stable thermal stability up to a temperature of 160.04°C. Four mass loss events were observed, the first between 169.28°C and 179.44°C, with a mass variation of 2.582%, the second between 187.75°C and 195.93°C, with a mass variation of 1.508%, the third

between 215.95°C and 229.38°C, with a mass variation of 9.516%, and the last event between 231.92°C and 322.40°C, with a mass variation of 52.62%. At the end of the heating process, the mass loss was 58.38%, leaving 41.62% of inorganic residue. The DTG curve showed three exothermic peaks: 176.83°C, 226.75°C and 288.76°C.

Furthermore, a decrease in the thermal stability of the binary mixture of SMZ and DOXY was also observed in the TG/DTG curves, when compared to the SMZ curve, as in isolated form this drug remained thermally stable up to 204.06°C.

The DSC curve of the mixture showed two endothermic peaks, the first between 141.81°C and 177.54°C with an enthalpy of 47.46 J g-1 and 160.12°C, Tonset of 168.40°C corresponding to DOXY melting, and between 178.21°C and 195.41°C, and between 178.21°C and 195.41 °C with enthalpy of 54.98 J g^{-1} and 180.36°C, Tonset of 187.60°C of SMZ fusion. The other peak observed in DSC was exothermic, at 215.02°C, attributed to sample crystallization.

Figure 29. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the binary mixture of sulfamethazine and doxycycline (1:1 m/m), obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



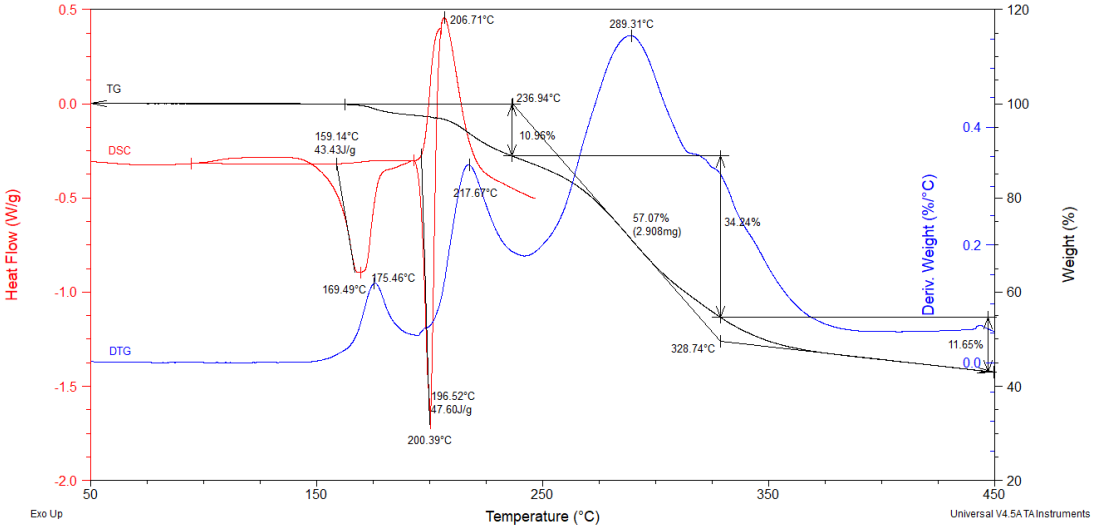
4.7.3 Binary Mixture of Trimethoprim (TMP) and Doxycycline (DOXY) (1:1 m/m)

In the TG/DTG curves (Figure 30), the mixture of TMP and DOXY was thermally stable up to a temperature of 164.34°C. Mass losses were observed in the range of 169.52°C to 180.64°C, with a variation of 2.456%, between 208.82°C and 225.31°C, with a variation of 9.132%, and at 236.94°C and 328.74°C, with a variation of 57.07%.

The total mass loss was approximately 64.95%, producing approximately 35.05% of residue. The DTG curve showed three exothermic peaks at 175.46°C, 217.67°C and 289.31°C.

In the DSC curve, two endothermic peaks were observed, between 140.91°C and 193.09°C, with T*onset* of 169.49°C, enthalpy of 43.3 J g^{-1} and 159.14°C, corresponding to the fusion of DOXY, and between 193.09°C and 204.44°C, enthalpy of 47.6 J g^{-1} and 196.52°C, with T*onset* of 200.39°C, related to TMP fusion. In addition to these peaks, an exothermic peak was also observed at 206.71°C, characteristic of crystallization.

Figure 30. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the binary mixture of trimethoprim and doxycycline (1:1 m/m), obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.

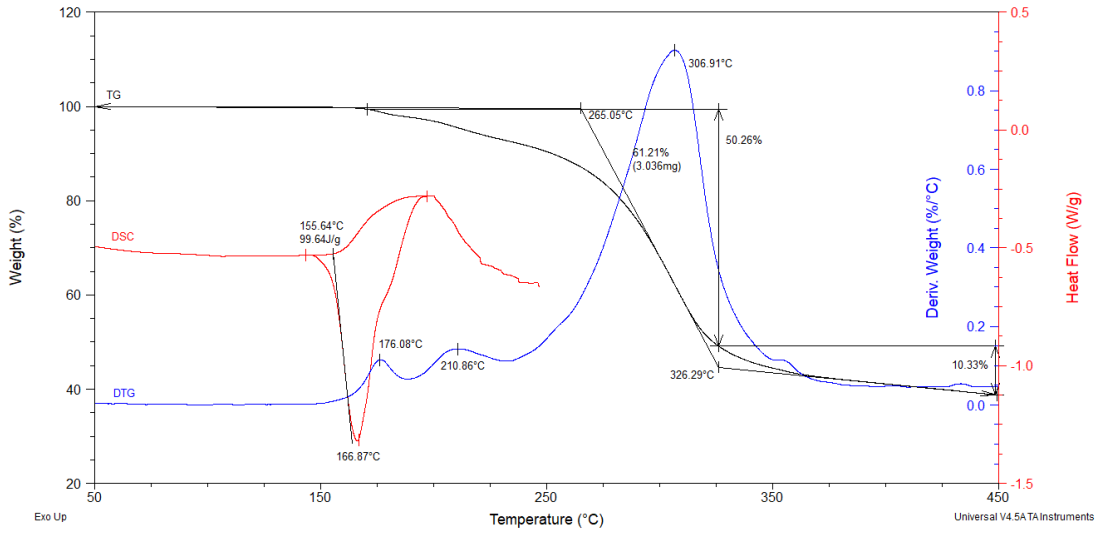


4.7.4 Ternary Mixture of Sulfamethazine (SMZ), Trimethoprim (TMP) and Doxycycline (DOXY) (1:1:1 m/m/m)

The TG/DTG curves of the SMZ + TMP + DOXY (Figure 31) mixture demonstrated stability up to a temperature of 166.92°C. Therefore, the ternary mixture presented lower thermal stability when compared to isolated drugs and binary mixtures. Three main mass loss events occurred: between 168.68°C and 180.16°C, with a mass variation of 1.995%, from 201.04°C to 220.10°C, with a mass variation of 4.582% and at 265.05°C and 326.09°C, with a mass variation of 61.21%. At the end of the heating process, the mass loss was 67.14%, leaving 32.86% of inorganic residue. The DTG curve showed exothermic peaks at 176.08°C, 210.86°C and 306.91°C.

The DSC curve of the mixture showed only one endothermic peak with an enthalpy of 99.64 J g^{-1} and 155.64°C, corresponding to the melting of the sample, with a T*onset* of 166.87°C. When comparing the DSC curves of SMZ, TMP and DOXY, isolated, with that obtained with the ternary mixture, it is possible to notice that there was a change in the thermal behavior, broadening and increase in the intensity of the peak corresponding to the melting.

Figure 31. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the ternary mixture of sulfamethazine, trimethoprim and doxycycline (1:1:1 m/m/m), obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.

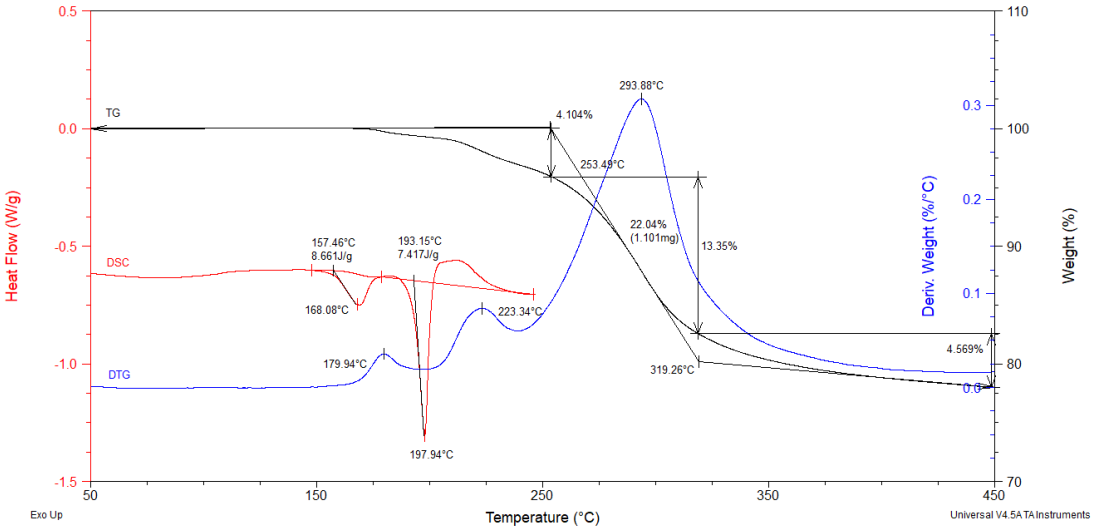


4.7.5 Commercial Sample

The TG/DTG curves of the commercial sample (Figure 32) demonstrated that it was thermally stable up to a temperature of 168.89°C. Three mass loss events occurred: between 172.49°C and 183.50°C, with a mass variation of 0.6544%, at 212.37°C to 228.68°C, with a mass variation of 2.055%, and between 253.49°C and 319.26°C, with a mass variation of 22.04%. At the end of the heating process, the mass loss was 73.47%, leaving 26.53% of inorganic residue. The DTG curve showed exothermic peaks at 179.94°C, 223.34°C and 293.88°C.

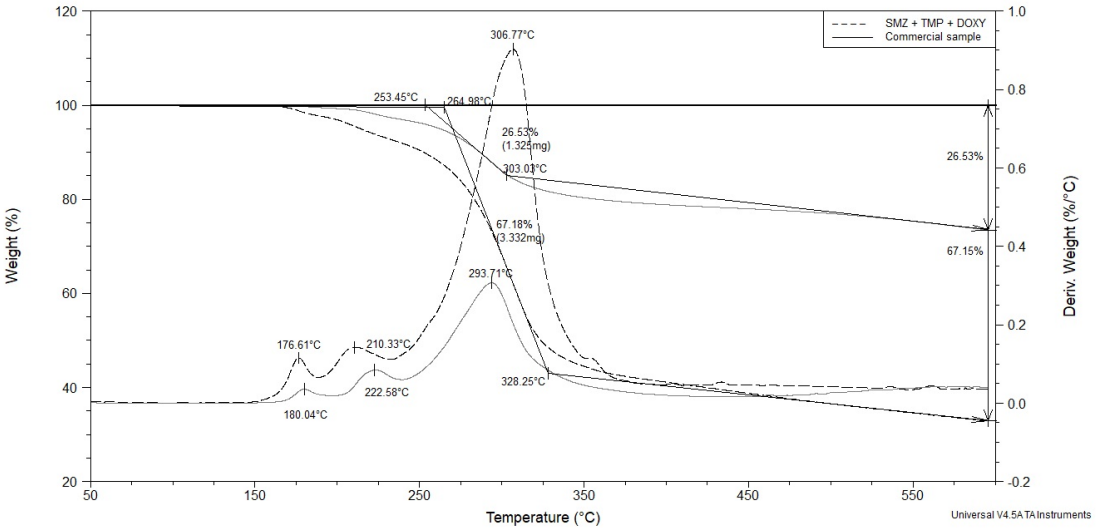
The DSC curve of the mixture showed two endothermic peaks: the first between 148.15°C and 179.23°C, with an enthalpy of 8.661 J g^{-1} and 157.46°C, and a T*onset* of 168.08°C, corresponding to the fusion of DOXY, and the second between 184.52°C and 208.76°C, with an enthalpy of 7.417 J g^{-1} and 193.15°C, and T*onset* of 197.94°C, suggesting the melting of the mixture of SMZ and TMP.

Figure 32. TG/DTG (flow rate of 40.0 mL min^{-1} up to 450ºC) and DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the commercial sample obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



When comparing the TG/DTG curves of the commercial sample and the ternary mixture (SMZ + TMP + DOXY), it was possible to observe that the profile obtained in the curves is similar, demonstrating mass loss in three consecutive stages (Figure 33).

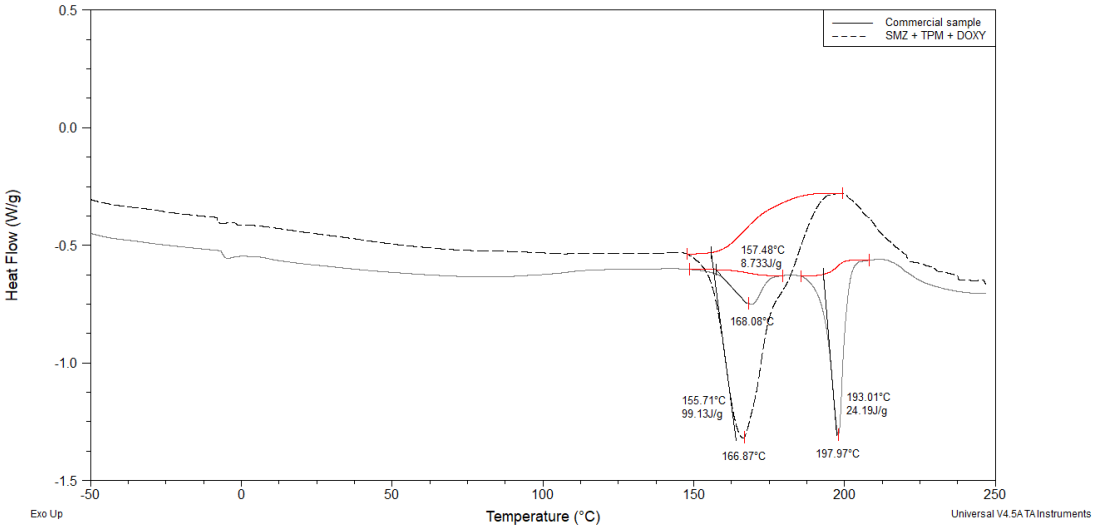
Figure 33. TG/DTG (flow rate of 40.0 mL min^{-1} up to 600ºC) curves of the commercial sample and ternary mixture of sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) (1:1:1 m/m/m) obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



When comparing the DSC curves of the commercial sample and the ternary mixture, it was observed that there was a considerable change in the thermal behavior of the ternary mixture in relation to the commercial sample. It is possible to notice that the commercial sample presented two exothermic peaks, while the ternary mixture curve

presented only one exothermic peak, with an increase in intensity and broadening (Figure 34).

Figure 34. DSC (flow rate of 60.0 mL min^{-1} and temperature range from -50 to 250°C) curves of the commercial sample and ternary mixture of sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) (1:1:1 m/m/m) obtained in a nitrogen atmosphere under a heating rate of 10ºC min^{-1}.



Furthermore, it was found that the commercial sample is more thermally stable than the ternary mixture, a fact that can be justified due to the presence of adjuvants in the formulation that may have increased its stability against increased temperature.

5. CONCLUSIONS

The qualification of sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) raw materials was carried out using chromatographic (TLC), spectroscopic (UV and IR) and thermal (TG, DTG and DSC) techniques. The qualitative results of the raw materials analyzes corroborated those already described in the literature.

No physical and chemical changes were identified in the drug molecules, allowing their use in the present study as characterized chemical substances (CCS) during the development of the analytical method.

The analytical method developed and validated by High Performance Liquid Chromatography (HPLC), for simultaneous quantification of SMZ, TMP and DOXY in medicines for animal use, could be considered indicative of stability, as it was possible to quantify the drugs in question without the interference of degradation products formed under stress conditions.

This method allowed the separation of drugs in an analysis time of less than 4 minutes, demonstrated to be linear in the concentration ranges for all drugs, with low detection and quantification limits, accurate with coefficients of variation below 2.0%, robust , showing no significant differences in the selected factors and variation levels used to carry out the analysis, accurate, with drug recovery values within the acceptance limits of 98 to 102%, and selective, without interference from adjuvants in the simultaneous determination of the drugs.

In the accelerated stability study, no major changes were observed in pH values, content and organoleptic analysis of the commercial sample.

Thermal analysis made it possible to verify the thermal stability of the raw materials and the commercial sample in the thermoanalytical curves. In this analysis, changes in the thermal stability of the drugs SMZ, TMP and DOXY were observed when combined in binary and ternary mixtures, when compared to the individual analysis of the drugs. Furthermore, the commercial sample was more stable than the ternary mixture of medicines.

The results highlight the need for the development and validation of analytical methods indicative of stability that can be used in the routine quality control of pharmaceutical industries, in order to guarantee the safety and effectiveness of products and medicines, whether for human or animal use.

6. CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

7. ACKNOWLEDGMENTS

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APPENDIX: PUBLISHED ARTICLE

Biomedical Chromatography

RESEARCH ARTICLE

Development and validation of method for the simultaneous determination of sulfamethazine, trimethoprim and doxycycline in veterinary formulation using high performance liquid chromatography

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Abstract

Sulfamethazine (SMZ), trimethoprim (TMP) and doxycycline (DOXY) are drugs of choice used in the treatment of intestinal and respiratory infections that affect poultry and swine. The aim of this study was develop and validate a simple, sensitive and fast method for the simultaneous determination of SMZ, TMP and DOXY in veterinary formulations by high-performance liquid chromatography. The separation was performed on a Macherey–Nagel C_8 analytical column (4 × 125 mm, 5 µm), with a flow rate of 0.5 ml min^{-1} and detection at 268, 270 and 350 nm, for SMZ, TMP and DOXY, respectively. All measurements were performed in acetonitrile–water (45:55 v/v; pH 3.0). The analytical curves were linear (r > 0.9997) in the concentration range of 5.0–35.0 µg ml^{-1} for SMZ, 1.0–7.0 µg ml^{-1} for TMP and 7.0–13.0 µg ml^{-1} for DOXY. The method proved to be precise, robust, accurate and selective. In accelerated stability, the sample was analyzed for 6 months, with no major variations observed in organoleptic analysis and pH. Therefore, the developed method was proved to be suitable for routine quality control analyses for the simultaneous determination of SMZ, TMP and DOXY in pharmaceutical formulations.

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