UNIVERSITY OF SÃO PAULO

FACULTY OF PHARMACEUTICAL SCIENCES

Graduation Program in Pharmacy – Pathophysiology and Toxicology

FERNANDA WAECHTER

Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil

São Paulo

2021

UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
Graduation Program in Pharmacy – Pathophysiology and Toxicology

FERNANDA WAECHTER

Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil

Corrected version

Dissertation presented to the Faculty of Pharmaceutical Sciences of University of São Paulo to obtain the title of Master of Science

Area: Pharmacy – Pathophysiology and Toxicology

Advisor: Prof. Elizabeth de Souza Nascimento
Co-advisor: Antonio Anax de Oliveira Falcão

São Paulo
2021

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

W126a

Waechter, Fernanda
Avaliação do risco de impurezas potencialmente mutagênicas em medicamentos anti-hipertensivos registrados no Brasil / Fernanda Waechter. - São Paulo, 2021.
100 p.

Dissertação (mestrado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Análises Clínicas e Toxicológicas - Programa de Pós-Graduação em Farmácia (Fisiopatologia e Toxicologia).
Orientador: Nascimento, Elizabeth de Souza
Coorientador: Falcão, Antonio Anax de Oliveira

1. impurezas farmacêuticas. 2. mutagenicidade. 3. ICH M7. 4. ensaios toxicológicos in silico. I. T. II. Nascimento, Elizabeth de Souza , orientador. III. Falcão, Antonio Anax de Oliveira , coorientador.

WAECHTER, F. **Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil.** 2021. Dissertation (Masters) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2021.

Aprovado em:

Banca Examinadora

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

Prof. Dr. _____

Instituição: _____

Julgamento: _____

To my parents, for their unconditional love, comfort, and support in all my decisions.

To my sister, who changed my world to a better place since the day she was born.

To my friend Simão Calvete Correa da Silva, who inspired me to go after my dreams and was always by my side in this journey.

To my inspiring co-advisor Antonio Anax Falcão de Oliveira, the responsible for lightening in me this will to learn more and be better.

To my dear advisor Prof. Elizabeth de Souza Nascimento, for receiving me at the University with such an open heart.

To Aché Laboratórios, for allowing me to go forward with the project that would change my professional life forever.

INDEX

RESUMO

WAECHTER, F. **Avaliação do risco de impurezas potencialmente mutagênicas em medicamentos anti-hipertensivos registrados no Brasil.** 2021. 100 p. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2021.

A presença de impurezas em medicamentos tem sido controlada conforme guias internacionais no Brasil e ao redor do mundo. Impurezas com potencial mutagênico tendem a apresentar uma maior toxicidade e consequentemente limites mais baixos do que os aplicados às demais impurezas. O guia internacional para controle de impurezas mutagênicas (ICH M7) não é aplicável a medicamentos já aprovados. Porém, recentemente algumas impurezas mutagênicas foram encontradas acima dos limites permitidos na valsartana, losartana, ranitidina, e outros medicamentos que já eram aprovados por agências reguladoras. O objetivo desse trabalho é identificar as possíveis impurezas mutagênicas em medicamentos anti-hipertensivos aprovados no Brasil e realizar a avaliação do risco propondo estratégias de controle para tais impurezas. As possíveis impurezas em cada fármaco foram identificadas com base no arquivo mestre do fármaco, um documento no qual o fabricante descreve o processo de fabricação e as impurezas potenciais do fármaco. Visando prever a mutagenicidade das impurezas, sistemas *in silico* foram utilizados, e para um caso inconclusivo o teste de Ames foi realizado. Para avaliar os níveis das impurezas no fármaco, foram utilizadas tanto ferramentas *in silico* (fator de purga), como métodos analíticos validados. Um total de 15 fármacos foi avaliado, e 262 impurezas foram identificadas. Os resultados mostram que 22% dessas impurezas são potencialmente mutagênicas, porém, com exceção das impurezas farmacopeicas, em todos os casos os níveis encontrados são inferiores aos limites aceitáveis. Isso sugere que apesar de haver a presença de impurezas mutagênicas em baixos níveis nos fármacos, os processos de fabricação atualmente utilizados parecem ser adequados para manter o risco negligenciável. É necessário estabelecer estratégias de controle que garantam que os níveis das impurezas mutagênicas permanecerão abaixo dos limites aceitáveis em todos os lotes produzidos. Além disso, recomenda-se a avaliação do potencial mutagênico de impurezas descritas em monografias farmacopeicas, a fim de definir se existe a necessidade de restrição dos limites.

Palavras – chave: impurezas farmacêuticas, mutagenicidade, ICH M7, ensaios toxicológicos *in silico*

ABSTRACT

WAECHTER, F. **Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil.** 2021. 100 p. Dissertation (Masters) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2021.

The presence of impurities in drug products has been controlled according to international guidelines in Brazil and the entire world. Impurities with mutagenic potential tend to show a greater toxicity and consequently need to be controlled to lower limits than the other regular impurities. The international guideline for control of mutagenic impurities (ICH M7) is not applicable for drug products which are already approved. However, some mutagenic impurities have recently been found above the permitted limits in valsartan, losartan, ranitidine, and other drug products which were already approved by regulatory agencies. The objective of this project is to identify the possible mutagenic impurities in anti-hypertensive drugs approved in Brazil and perform the risk assessment proposing control strategies for such impurities. The possible impurities in each drug substance were identified based on the drug master file, a document where the manufacturer describes the manufacturing process and potential impurities of the drug substance. For the mutagenicity prediction of the impurities, *in silico* systems were used, and in one inconclusive case the Ames test was performed. For evaluation of the levels of the impurities in the drug substance, *in silico* tools such as the purge factor approach, as well as validated analytical procedures were used. A total of 15 drug substances was evaluated, and 262 impurities were identified. The results show that 22% of these impurities are potentially mutagenic, however all of them, except for impurities described in a pharmacopoeial monograph, are below the acceptable limits. This suggests that although mutagenic impurities are present at low levels in drug substances, the current manufacturing processes seem to be adequate to keep the negligible risk. Nonetheless, there is an evident need to establish control strategies which ensure the levels of mutagenic impurities are below the acceptable limits throughout all manufactured batches. Moreover, it is recommended to evaluate the potential mutagenicity of impurities described in pharmacopoeial monographs, understanding whether there is a need to tighten limits.

Key words: pharmaceutical impurities, mutagenicity, ICH M7, *in silico* toxicological assays

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Anvisa	Agência Nacional de Vigilância Sanitária (Brazilian Sanitary Vigilance National Agency)
API	Active pharmaceutical ingredient
CTD	Common technical document
DMF	Drug master file
DNA	Deoxyribonucleic acid
ECHA	European Chemicals Agency
EP	European Pharmacopoeia EP
FDA	Food and Drug Administration
GMP	Good manufacturing practice
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
OECD	Organization for Economic Co-operation and Development
TD_{50}	Tumor dose for 50% of individuals
TTC	Threshold of toxicological concern

1 INTRODUCTION

1.1 PROBLEM ELUCIDATION

The presence of impurities in drug substances is inevitable, because no route of synthesis can generate a 100% pure drug substance. Reagents, solvents, catalysts, and by-products can be carried over to the final drug substance during the manufacturing process. Moreover, the drug substance and drug product can degrade during their shelf life and generate new impurities.

Guidelines published by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) regulate and limit the presence of such impurities in drug substances and drug products (ICH Q3A (R2), 2006; ICH Q3B (R2), 2006; ICH Q3C (R6), 2016; ICH Q3D (R1), 2019). However, some impurities may be mutagenic, presenting a higher toxicological concern, and are not covered by such guidelines. Only in 2014 a specific guideline on mutagenic impurities was published, the ICH M7, which was further revised in 2017. This guideline establishes lower acceptable limits for these impurities, aiming to limit the carcinogenic risk posed by them (ICH M7 (R1), 2017). However, ICH M7 is not applicable to drug products which are already approved by regulatory agencies. It only applies to new drug products being developed or when there are significant changes to already approved products, which could affect their impurity profile.

Nevertheless, in July 2018, a mutagenic and carcinogenic impurity was identified in batches of valsartan, which was a drug substance already approved, produced by a Chinese company (EMA, 2018). This impurity, N-nitrosodimethylamine (NDMA) was only the first of many nitrosamines investigated in angiotensine II receptor antagonists, the so-called ‘sartans’. This investigation led to the worldwide recall of ‘sartans’ batches (FDA, 2018), including in Brazil (ANVISA, 2019a), as well as the publication of RDC 283/2019, which is a Brazilian regulation for the investigation, control and elimination of potentially carcinogenic nitrosamines in angiotensin II receptor antagonists. The need to investigate nitrosamines was later expanded to all drug products on the market (EMA, 2019a), after some nitrosamines were found in non-sartan drug products such as ranitidine and metformin (EMA, 2019b, 2019c), indicating this was a much broader issue.

Considering this scenario, one may wonder how were these drug products even approved, if they contain such toxic impurities? In order to get the regulatory approval of a new drug product, the efficacy and safety must be demonstrated through pre-clinical and clinical trials, so that therapeutic and side effects can be identified. However, studies performed before the marketing of the drug have restrictions regarding the number of volunteers and duration of exposure. Hence, many of these effects are only observed after the drug is approved, when a greater part of the population is exposed to the compounds for longer periods of time. For monitoring the side effects after the drug product is on the market, the pharmacovigilance is used (LÜLLMANN, 2008).

The development of cancer is an example of a long-term side effect, which should be detected and related to the use of drug products through pharmacovigilance studies. However, cancer is a multifactorial disease, which can have multiple different causes. Some examples of carcinogens are the lifestyle (food, tobacco), environmental factors such as pollution, ultraviolet rays, and drug products (AMERICAN CANCER SOCIETY, 2016). This poses a challenge in relating a new case of cancer to a specific cause such the use of a certain drug product.

Therefore, specific studies should be performed in early drug development to mitigate the risk of cancer. The standard non-clinical carcinogenicity study is a 2-year *in vivo* assay where the chemical is administered to animals to verify whether tumors are formed. However, besides being expensive and time-consuming (ICH S1A, 1996; OECD, 2011), avoidance of the use of animals in research is recommended. Hence, more simple assays targeting the mechanisms which lead to cancer can be helpful to understand a potential risk of cancer.

The carcinogenesis mechanisms can be genotoxic or non-genotoxic. Genotoxic mechanisms are caused by direct interactions with the DNA, while non-genotoxic mechanisms include metabolic changes, alterations in the immune system, as well as epigenetic mechanisms, which act indirectly by affecting the expression of the DNA (PLOŠNIK; VRAČKO; DOLENC, 2016). Genotoxicity is a broad term which refers to any damage to the DNA, including formation of DNA adducts, chromosomal damage, chromosomal breakage, or aneuploidy. The human body has intrinsic DNA repair mechanisms, so a genotoxic compound may not always lead to a mutation. A mutation is a permanent and heritable change in the DNA, which can result from the DNA damage in case it is not repaired or is repaired inefficiently. Hence, mutagenicity refers

to the capacity of a compound to cause a mutation in the DNA, which could be the first step for the development of cancer. However, for cancer to develop, more than one mutation is usually necessary (MÜLLER et al., 2006; PECORINO, 2012; PLOŠNIK; VRAČKO; DOLENC, 2016).

Although many different mechanisms are possible, the mutagenicity endpoint is the only one relevant for impurities which are present at low levels in drug substances to limit their carcinogenic risk. This is because this endpoint has a linear dose-response relationship, so the compounds can pose a risk even at low levels. Agents which can cause cancer through indirect mechanisms, on the other hand, usually act through threshold mechanisms, so that they only pose a risk above a certain level (AMES; LOIS; GOLD, 1990). Hence, considering the low levels in which impurities are found in drug substances, these threshold mechanisms are not relevant for them (MÜLLER et al., 2006). Due to its high correlation with the outcome of carcinogenicity, and the amount of data already available, the Ames test has been widely used and internationally recommended even when assessing the carcinogenic risk posed by impurities (EMA, 2006).

The genotoxicity evaluation of drug substances is already included in the pre-clinical protocols for registering a new drug product (ICH M3 (R2), 2008). However, before publication of ICH M7, there was no international requirement for evaluation of the genotoxic potential of impurities present in the drug product. This explains why some products may have been approved without such assessment.

However, the presence of a mutagenic impurity in a drug substance may not always be a problem. According to one of the basic principles of Toxicology, set by Paracelsus (1493-1541), all substances can be considered toxic or safe, the dose makes the poison (HAYES; KRUGER, 2014). This means that the toxic effects related to the presence of impurities in drug substances can be limited if the impurities do not exceed the levels proven to be safe. The risk assessment for potentially mutagenic impurities in drug substances and drug products is described in ICH M7 and follows these four steps: hazard identification, dose-response evaluation, exposure evaluation and risk characterization (NRC, 2007).

1.2 HAZARD IDENTIFICATION

The hazard identification is the first step of the risk assessment and consists of identifying whether the impurity is mutagenic or not. While the traditional and most widely accepted experimental test for mutagenicity is the Ames test, ICH M7 recommends the use of *in silico* methodologies for prediction of the mutagenic potential of the impurity, specifically for point mutations in bacteria, when the Ames test result is not available. These methods perform an analysis of the relationship between the chemical structure and the biological activity and are hence called (Q)SAR - (quantitative) structure-activity relationship methods. Using a database of compounds which have already been tested in the mutagenicity assay, associated with their results, the software can generate predictions for new compounds based on their structural similarity to the compounds in the database (SUTTER et al., 2013). For predicting the mutagenic potential of impurities, ICH M7 recommends the use of two complementary *in silico* systems – one expert-rule based, and one statistical based. After the prediction, an expert review must be performed and the result is then sufficient to establish the mutagenicity or not of the impurity (ICH M7 (R1), 2017).

Lhasa Limited is a not-for-profit company which developed *in silico* methods that comply with ICH M7 criteria for the mutagenicity prediction. Derek Nexus is an expert-based system for prediction of mutagenicity, while Sarah Nexus is a statistical-based system (LHASA LIMITED, 2018). These tools are available for regulatory agencies and can be acquired by academic institutions and pharmaceutical companies worldwide.

Derek (Deductive Estimation of Risk from Existing Knowledge) in its latest version (6.1.0) includes 133 structural alerts for the mutagenicity of compounds (HASSELGREN et al., 2020), using a database built with public and proprietary data donated by regulatory agencies and pharmaceutical companies. This software is based on rules created by experts, which include information on reactivity, metabolism and toxicity. When the query structure is drawn on the software, toxicophores are identified and compared to the structural alerts of the database and their correlation with positive results for mutagenicity. Then a confidence in the prediction based on a reasoning level is generated that the structural alert will lead to the mutagenicity of the studied compound (GREENE et al., 1999).

Sarah Nexus contains a database of over 10,000 molecules associated with their results in the Ames test. This software is different from Derek, because it is based on machine learning. The compounds which are part of the database have their structures fragmented by the software, and each fragment is associated with the mutagenicity result of the compound which originated it. When the query compound is then drawn in the software, the system creates positive or negative hypotheses for the compound, based on the similarity with a series of molecules and their mutagenicity result. A level of confidence is established for the prediction of each hypothesis, based on the structural similarity of the query compound with the nearest neighbors from the database. Finally, the confidence levels for all predictions are combined to obtain the final prediction for the mutagenic potential of the compound, which can be positive, negative, equivocal or out of domain, in a confidence scale of 0 to 100% (BARBER et al., 2016).

Sarah Nexus was developed in a research cooperation agreement with the FDA (Food and Drug Administration) and, in combination with Derek Nexus, complies with the necessary criteria for the *in silico* evaluation of the mutagenic potential of impurities, as defined in ICH M7.

As a result of the *in silico* prediction for the mutagenic potential, and considering mutagenicity and carcinogenicity experimental data if available, each impurity is classified in one of five classes defined in ICH M7, according to Table 1. For each class a specific action for control is proposed, based on the risk. Classes 1 and 2 correspond to mutagenic impurities, and classes 4 and 5 are impurities considered to be non-mutagenic. Class 3 impurities should be controlled as mutagenic, as a conservative approach, or tested through the Ames test as an attempt to refute the mutagenicity prediction (ICH M7 (R1), 2017).

Table 1. Classification of impurities based on the mutagenic and carcinogenic potentials and proposed actions for control.

Class	Definition	Proposed action for control
1	Known mutagenic carcinogens	Control at or below compound-specific acceptable limit
2	Known mutagens with unknown carcinogenic potential (bacterial mutagenicity positive, no rodent carcinogenicity data)	Control at or below acceptable limits (appropriate TTC)
3	Alerting structure, unrelated to the structure of the drug substance; no mutagenicity data	Control at or below acceptable limits (appropriate TTC) or conduct bacterial mutagenicity assay;

		If non-mutagenic = Class 5 If mutagenic = Class 2
4	Alerting structure, same alert in drug substance or compounds related to the drug substance (e.g. process intermediates) which have been tested and are non-mutagenic	Treat as non-mutagenic impurity
5	No structural alerts, or alerting structure with sufficient data to demonstrate lack of mutagenicity or carcinogenicity	Treat as non-mutagenic impurity

Although *in silico* systems are mentioned by ICH M7 and these have also been widely validated, it is recommended that a critical evaluation of the predictions be performed by an expert, to verify if they are in fact reliable and adequate (BARBER et al., 2015; ICH M7 (R1), 2017). In some cases, the expert-rule based and statistical based systems do not agree with each other, or they may lead to inconclusive results instead of classifying the impurity within classes 1-5. In these cases, the expert review is especially important in order to assign a class to the impurity (AMBERG et al., 2019).

1.3 DOSE-RESPONSE EVALUATION

The dose-response evaluation is performed to understand which doses cause the toxic effect and to establish acceptable levels of exposure for the impurity.

For mutagenic impurities in drug substances, the acceptable limits have been proposed by ICH M7 based on the TTC (threshold of toxicological concern), which corresponds to the intake, expressed in µg/day, below which a given compound is not expected to present a toxicological concern. The TTC for mutagenic impurities in pharmaceuticals is 1.5 µg/day/person, which represents an exposure which will cause a negligible risk of cancer throughout a lifetime.

This value was derived from a study where more than 700 compounds were associated with their carcinogenic potential, from the extrapolation of the dose which caused a tumor incidence in 50% of the cases to an incidence of 1 in 10^6 individuals. The value obtained in this study was 0.15 µg/day, which is the level at which there is 86-97% probability that the intake of a carcinogenic compound would correspond to a cancer risk of 1 in 10^6 (KROES et al., 2004). Since pharmaceuticals present a benefit to the patients, an acceptable intake of 1.5 µg/day, representing a risk of 1 in 100,000

cases, was considered acceptable for mutagenic impurities arising from drug substances (EMA, 2006).

Hence, whenever the impurity was classified as class 2 or 3, which means that it is potentially mutagenic, but no carcinogenicity data is available, the limit in the drug substance was derived from the TTC. In order to establish a limit for the impurity in the drug substance, the TTC is divided by the maximum daily dose of the drug substance, which represents the highest exposure that is expected for the patient using that drug product.

Class 1 impurities, on the other hand, are known carcinogens, hence a specific limit can be derived from the carcinogenicity study, instead of using a standard value. The same rationale applied to obtain the TTC is used for class 1 compounds, so the TD_{50} – dose causing a cancer incidence of 1:2 – is divided by 50,000 to yield the dose which causes a cancer incidence of 1:100,000. Since the TD_{50} is usually reported in mg/kg/day, to account for the average human body weight the result must also be multiplied by 50 kg. The acceptable intake resulting from this calculation is then divided by the maximum daily dose of the drug substance.

ICH M7 also describes some classes of compounds for which – although carcinogenicity data may not be available - the TTC would not be applicable, since they are more potent than the majority of carcinogens. This is described as the cohort of concern, which consists of aflatoxin-like, N-nitroso, and alkyl-azoxy compounds. When establishing the limit for these impurities, if carcinogenicity data is not available, the recommendation is to compare the structure with other compounds for which carcinogenicity data is available (ICH M7 (R1), 2017).

Class 4 and 5 impurities are considered non-mutagenic and are thus regulated by the ICH guidelines for impurities in new drug substances (Q3A) and drug products (Q3B).

1.4 EXPOSURE EVALUATION

After defining the acceptable levels, the exposure evaluation must be done to measure the intensity, frequency and duration of human exposure to the toxic agent. Since mutagenic impurities are present in drug substances, which are then consumed by the patient, the exposure is measured considering the daily dose of the drug taken

by the patient as well as the concentration of the impurity present in the drug substance. This evaluation is traditionally done using analytical procedures to experimentally determine the amount of impurity in the drug substance. These procedures must be validated for this purpose and must show adequate sensitivity considering the low limits usually established for mutagenic impurities (ICH M7 (R1), 2017).

Another possibility to estimate the level of the impurity in the drug substance, which is also an alternative presented by ICH M7, is the purge factor approach. This can be applied to impurities arising from the synthesis of the drug substance and consists of a calculation that shows the extent to which the manufacturing process is capable of eliminating the impurity. Since many stages are performed throughout the process to synthesize and purify the drug substance, and considering that the impurity and the drug substance may have different properties – reactivity, solubility, volatility – this approach is based on the ability of the process stages to separate the impurity from the drug substance. For example, if the impurity has a lower boiling point than the drug substance, a drying stage with a temperature higher than the impurity's boiling point is capable of volatilizing the impurity while keeping the drug substance in the medium, hence eliminating the impurity. Work up stages such as extraction, centrifugation, or filtration, for example, could purge and eliminate the impurity through differences in solubility when compared to the drug substance (TEASDALE et al., 2010).

This scientific-based approach used to understand how much the process purges an impurity has been used by the pharmaceutical industry to justify elimination of mutagenic impurities and has been accepted by regulators.

In order to standardize how these calculations are done, some recommended values have been published for each purge, which usually underestimate the purges, due to the conservative nature of this approach (TEASDALE et al., 2013). For example, if a drying stage uses a temperature 20ºC higher than the impurity's boiling point, a purge of 10 can be given for this stage. If in the sequence there is a washing stage with a solvent where the impurity is highly soluble, but the drug substance is insoluble, once again a purge of 10 can be given. The overall predicted purge factor is the multiplication of the individual ones. Therefore, in this case, these two stages can reduce the concentration of the impurity by 100 times. This means that if the initial

concentration of the impurity was 1000 ppm, after these two stages the theoretical final concentration would be 10 ppm.

After obtaining the predicted purge factor, to know if it is enough to reduce the impurity to levels below its safe limit, it must be compared to the required purge factor, which is essentially the initial concentration of the impurity divided by its safe limit. The ratio of the predicted purge factor divided by the required purge factor (purge ratio) is an estimation of how much the process can in fact reduce the impurity effectively, ensuring it would not exceed the acceptable limit if tested (BARBER et al., 2017).

An *in silico* tool called Mirabilis has been developed by Lhasa Limited to help estimating the purge factor. This software was built by a consortium of experienced chemists and contains a knowledge base of reactions and their association with specific classes of compounds, hence giving automatic predictions for reactivity factors if the structure and reaction are within the knowledge base (BURNS et al., 2019).

1.5 RISK CHARACTERIZATION

In the last stage, the risk characterization consists of comparing the calculated safe limits with the daily exposure to the toxic agent (NRC, 2007). This will finally indicate the actual risk posed by mutagenic impurities to the patients.

After the risk assessment has been concluded, ICH M7 establishes that a control strategy must be defined for any potentially mutagenic impurity identified in the manufacturing process of the drug substance, in order to ensure that the risk assessment will be sustainable throughout all manufactured batches.

These control strategies can either consist of analytical controls, by using validated procedures with adequate sensitivity for the expected limits, or process controls, which must ensure the purge of the impurity (ICH M7 (R1), 2017). The guideline recommends four possibilities of control strategies that can be adopted, as detailed in Table 2.

Table 2. Control strategies for potentially mutagenic impurities and requirements.

	Control strategy	Requirement
Option 1	Control the impurity in the quality control specification of the drug substance, using the acceptable limit defined in the guideline. In case consecutive batches results	A change in the drug substance specification has regulatory impact in Brazil, hence it requires a variation procedure as per RDC 361/2020.

	demonstrate the absence of the impurity – results below 30% of the acceptable limit – it may be possible to establish periodic testing instead of routine testing.	
Option 2	Control the impurity in the quality control specification of an intermediate of the process, using the acceptable limit defined in the guideline.	The impurity cannot be further generated in the process after the intermediate where its control is established. A change in the specification of the intermediate has regulatory impact in Brazil as per RDC 361/2020.
Option 3	Control the impurity in the quality control specification of an intermediate of the process, using a limit higher than the acceptable limit defined in the guideline.	It must be demonstrated that the stages following the intermediate where the control is established will be able to purge and eliminate the impurity, ensuring its level in the drug substance will be below the acceptable limit defined in the guideline. The regulatory impact of changing the specification of an intermediate must also be considered as per RDC 361/2020.
Option 4	Process control (purge factor) – no analytical control in any specification	It must be demonstrated that the stages following the generation of the impurity will be able to purge and eliminate it, ensuring its level in the drug substance will be below the acceptable limit defined in the guideline.

Options 1, 2 and 3 represent traditional strategies which have been adopted by the industry for a long time. Besides these options, ICH M7 outlines a new concept of controlling impurities, which is option 4. This option relies on the purge factor calculation. According to a publication by Barber and colleagues, option 4 can be used if the purge ratio is higher than 1 – meaning that the predicted purge factor is higher than the required purge factor – as long as additional data be provided as needed (BARBER et al., 2017). Table 3 explains which supporting data would be recommended based on this publication, to support the use of option 4, depending on the purge ratio result.

Table 3. Purge ratio and its relationship with the control strategy proposed by Barber and colleagues (2017) (BARBER et al., 2017)

Purge ratio	Definition
>1000	A purge 1000 times more than it would be needed is predicted, indicating that no additional test is necessary to justify option 4.
100-1000	The predicted purge is between 100 and 1000 times more than it would be needed. In these cases, option 4 can be justified but it is recommended to present supporting information for key purges, such as non-trace analytical data - solubility measurements or HPLC data from reaction monitoring for example.
1-100	The predicted purge is higher than the required purge, however to support the use of option 4, stronger evidence should be provided to confirm the calculation, such as spike and purge studies – adding known amounts of the impurity to a stage and measuring it in a later-on stage, to confirm how much the process has purged.
<1	In this case the purge factor does not demonstrate the adequate elimination of the impurity, and one of the other control options must be used – 1, 2 or 3.

The risk assessment for potentially mutagenic impurities in drug substances is well established in ICH M7, although it is not a regulatory requirement for approved drugs. However, the contaminations with nitrosamines are an example of the relevance of applying ICH M7 principles to drug products which are already on the market.

In this project, impurities arising from already approved drug substances were evaluated. The impurities were identified based on the manufacturing process, impurity section and specification described by the drug substance manufacturer in the drug master file (DMF). The mutagenic potential was assessed using *in silico* tools as recommended by ICH M7, and the risk assessment was performed to conclude if control measures should be established.

2 JUSTIFICATION

The international guideline for control of mutagenic impurities to limit carcinogenic risk, ICH M7, is only applicable to new drug products, or when there is a significant change in approved drug products, affecting their impurity profile. For drugs which are already approved, the controls should be established only if known mutagenic or carcinogenic compounds are present. However, the recent findings of carcinogens in losartan, valsartan, ranitidine, and other approved drugs raised the question of whether there should be a concern with those drugs which are already on the market or not.

The choice of drug substances to be studied was made considering the relevance to public health. According to the Pharmaceutical Market Statistical Yearbook, published in 2016 by Anvisa (Brazilian Sanitary Vigilance National Agency), the most widely commercialized drugs in Brazil have an indication for cardiovascular diseases. The therapeutic classes involved are antiarrhythmic, vasodilator, cardiotonic, anti-hypertensive drugs, among others (CMED; ANVISA, 2017).

The class chosen to be evaluated is the anti-hypertensives, considering that the first alerts raised by nitrosamines were in ‘sartans’, which are a class of anti-hypertensive drugs, and the fact that these drugs are for chronic use, which cause a higher concern due to the long-term exposure to the drug and related impurities.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

The objective of this project is to identify the possible mutagenic impurities described in the drug master file of anti-hypertensive drug products approved in Brazil, and to perform the risk assessment proposing control strategies for such impurities.

3.2 SPECIFIC OBJECTIVES

- Identify the possible impurities in anti-hypertensive drug substances, based on the analysis of their routes of synthesis;

- Identify the potentially mutagenic impurities through two *in silico* methodologies as recommended in ICH M7;

- Evaluate the risk, verifying the elimination or need to establish control strategies for the potentially mutagenic impurities which were identified.

4 MATERIAL AND METHODS

4.1 IDENTIFICATION OF IMPURITIES

A total of 15 anti-hypertensive drug substances were evaluated regarding their route of synthesis and possible impurities. All evaluated drug substances are used in drug products which are already approved and currently marketed in Brazil. There are many subclasses of anti-hypertensives. The evaluated drugs were mainly beta-blockers, angiotensin II receptor antagonists, diuretics, and calcium channel blockers. This project is a partnership with Aché Laboratórios, which is a pharmaceutical company, so the drugs selected for the evaluation were all the anti-hypertensive drugs currently commercialized by this company.

The evaluation was based on the drug master file (DMF), a document issued by the drug substance manufacturer which is shared with its customers and regulatory agencies. The pharmaceutical company has access to the DMFs of the drug substances used in its drug products, and since this is a confidential document, a confidential disclosure agreement has been signed between the pharmaceutical company and the drug substance manufacturer. Hence, the information presented in the DMF has not been disclosed in this project, only used for the assessment.

The DMF is usually organized in the Common Technical Document (CTD) format, where section 3.2.S.2.2 consists of the description of the manufacturing process, section 3.2.S.3.2 consists of a discussion of impurities and their control, and section 3.2.S.4.1 presents the specification for the routine quality control of the drug substance (ICH M4Q (R1), 1992). These three sections have been analyzed to list all potential impurities related to the drug substance. Any starting material, intermediate or reagent described in the manufacturing process was considered a potential impurity, as well as any impurity discussed by the manufacturer in the impurity section or controlled in the specification.

For some of the assessed drug substances, more than one manufacturer was evaluated, totalizing 22 DMFs (that is, 22 manufacturing processes) evaluated.

4.2 MUTAGENICITY ASSESSMENT

Each impurity identified had its mutagenicity predicted using the ICH M7 classification functionality within the Nexus platform, which integrates both Derek (expert rule-based) and Sarah (statistical-based) predictions. The settings used are presented in Table 4.

Table 4. Settings used for mutagenicity prediction through the ICH M7 functionality for Derek and Sarah Nexus systems.

	SYSTEM	
PARAMETERS	**Derek Nexus**	**Sarah Nexus**
Software version	6.0.1	3.0.0
Endpoint	In vitro mutagenicity	In vitro mutagenicity
Species	Bacteria	Bacteria
Knowledge base	Derek KB 2018 1.1	-
Model	-	Sarah Model - 2.0
Hypothesis method	-	Weighed
Equivocal level	-	8%
Sensitivity level	-	8%

All predictions were run before the release of the latest version of Derek and Sarah Nexus, however the mutagenicity predictions of two cases (Atenolol impurity D which had its prediction compared to the Ames test result, and nitrendipine impurities for which an analytical procedure was developed) were repeated in the updated version of the models, using the settings as described in Table 5.

Table 5. Settings used for mutagenicity prediction of atenolol impurity D and nitrendipine impurities.

	SYSTEM	
PARAMETERS	**Derek Nexus**	**Sarah Nexus**
Software version	6.1.0	3.1.0
Endpoint	In vitro mutagenicity	In vitro mutagenicity
Species	Bacteria	Bacteria
Knowledge base	Derek KB 2020 1.0, version 1.0	-
Model	-	Sarah Model – 2020.1, version 1.8

Hypothesis method	-	Weighed
Equivocal level	-	8%
Sensitivity level	-	8%

Although the latest version of the models was not used for most predictions, it is considered unnecessary to repeat the predictions after the release of the updated model, based on a study that showed that most updates in *in silico* models for mutagenicity predictions correspond to positive predictions changing to negative (HASSELGREN et al., 2020). Hence, it is not expected that re-running the predictions would increase the number of positive results – i.e. impurities considered to be potentially mutagenic which would need to be included in the risk assessment - so the original predictions are considered valid.

In this study, each prediction was followed by an expert review, which was usually an evaluation of the additional information provided by the *in silico* systems. Derek is a tool which presents additional information based on the literature for each presented alert. These details were closely evaluated to understand the mechanisms of mutagenicity for that specific alert, as well as their applicability to the query impurity.

Sarah, on the other hand, generates hypotheses which are related to the fragments of the compound, and presents examples which are within the database for each hypothesis, along with their respective result in the mutagenicity assay – positive or negative. In the expert review, the examples shown by Sarah were evaluated to understand if they were relevant to the query. Many times, the examples shown by Sarah, although sharing one specific fragment of the structure with the query, also present other mutagenicity alerts in their structure, which may not be present in the query. Such examples were evaluated in order to assess whether the prediction given by Sarah was relevant or not.

For cases giving inconclusive or out-of-domain results, the expert review was complemented with literature research, in line with the approach recently described and recommended for such cases (AMBERG et al., 2019). One example of a database used for this research was the ECHA (European Chemicals Agency) website where registration dossiers for various compounds are presented. This platform usually

reports the results for mutagenicity and carcinogenicity studies in which the applicant relied upon to reach a conclusion regarding the toxicity of the compound.

Depending on the results of the expert review, the class given automatically by the *in silico* systems may have been changed. Inconclusive or out-of-domain predictions were ultimately placed into one of the five classes. For these cases, when the weight-of-evidence was not enough to classify an impurity as non-mutagenic, a class 3 was assigned.

In addition, since anytime that Derek Nexus presents a positive prediction for mutagenicity, the specific structural alert responsible for this prediction is described by the software, in this project the Derek alerts have been evaluated, in order to identify if any specific alert is more common than others.

4.3 RISK ASSESSMENT

For impurities assigned to classes 1, 2 or 3, the risk assessment was continued, since they present a mutagenic potential. The acceptable limit was calculated based on ICH M7, using the TD_{50} for class 1 impurities and the TTC for class 2 and 3 impurities.

The evaluation of exposure was performed using both experimental and predictive methods – analytical procedures and the purge factor. This stage was performed upon evaluation of the information presented in the DMF and through contacting the drug substance manufacturer in case this specific information was not originally presented. When analytical results were already available at the drug substance manufacturer end, these were evaluated as well as the corresponding analytical validation, which must be performed based on ICH Q2 (ICH guideline for validation of analytical procedures) principles.

If analytical results were not yet available, the purge factor was calculated as described by Teasdale and colleagues (TEASDALE et al., 2013), either manually or using Mirabilis (LHASA LIMITED, 2018). However, since the purge factor is a prediction, it was also performed for one case where analytical results were available, so that both results could be compared as an additional way to verify the reliability of the prediction. The purge factor gives an estimate of the level of the impurity that would

be expected in the drug substance, and this was compared with the level quantified by a suitable and validated analytical method.

Besides the comparison of the purge factor with analytical results, a mutagenicity prediction was also compared with the experimental result of the Ames assay for one impurity.

Once the risk assessment was concluded, the control strategy for each impurity was evaluated. If such information was not available in the DMF presented by the drug substance manufacturer, the manufacturer was contacted and requested to share this specific information.

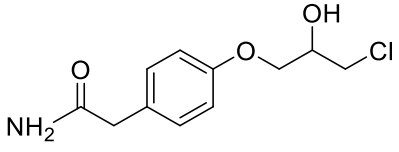
4.4 THE AMES TEST FOR ATENOLOL IMPURITY D

The Ames test was performed for one impurity which presented a positive prediction in the *in silico* mutagenicity assessment, originally assigned to class 3. The test was done at Bioneeds India Private Limited, in compliance with the OECD Principles of Good Laboratory Practice (GLP) and in accordance with the OECD guideline for testing of chemicals No. 471 “Bacterial Reverse Mutation Test”, adopted on 21st July 1997.

4.4.1. Test article

The compound 2-[4-[(2RS)-3-chloro-2-hydroxypropoxy] phenyl]-acetamide (hereby called Atenolol Impurity D – Figure 1) used in these studies was supplied by Kopran Research Laboratories Limited as off-white crystals at a purity level of 85.65% and was dissolved in dimethyl sulfoxide (DMSO) for the bacterial reverse mutation test, in order to prepare the appropriate testing concentrations. Due to the relatively low purity of the compound, its impurity profile was evaluated, which confirmed that the remaining 14.35% corresponded to impurities not presenting any mutagenicity alert that could interfere with the assay.

Figure 1. Structure of Atenolol impurity D



Impurity D

4.4.2. Test system

The tester strains used were *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101) and were obtained from Molecular Toxicology, Inc. PO Box 1189 Boone, NC 28607 USA. Each of the tester strains from the master plates were grown in oxoid nutrient broth no. 2. A fresh culture of bacteria was grown up to late exponential or early stationary phase of growth. The inoculum was incubated in a water bath at 37±1ºC for 15 hours and 35 minutes for initial cytotoxicity and 15 hours and 25 minutes for plate incorporation and preincubation methods. The inoculum was adjusted to a density of 18×10^8 cells/ml.

4.4.3. Preparation of S9 mix

Sodium phenobarbitone and β-naphthoflavone induced rat liver S9 homogenate was used as the metabolic activation system. The S9 homogenate was prepared from male Wistar rats induced with intraperitoneal injection of sodium phenobarbitone and β-Naphthoflavone at 16 mg/ml and 20 mg/ml, respectively, for 3 days prior to sacrifice. The S9 homogenate was prepared and stored in the test facility at -80±10ºC until use. Each batch of S9 homogenate was assessed for sterility by streaking the supernatant fluid on Nutrient Agar plates and incubated at 37±1ºC for 24 hours. It was found sterile and was further evaluated for its protein content (Modified Lowry Assay, Sword and Thomson, 1980) and for its ability to metabolize the promutagens 2-Aminoanthracene and Benzo(a)pyrene to mutagens using *Salmonella typhimurium* TA100 tester strain. The results were found to be acceptable for the tested parameters.

A volume of 1 ml of S9 homogenate was thawed immediately before use and mixed with 9 ml of co-factor solution containing 4 mM Nicotinamide Adenine Dinucleotide Phosphate (NADP) disodium salt, 5 mM Glucose-6-phosphate, 8 mM MgCl2 and 33 mM KCl in Phosphate Buffer Saline (PBS) of pH 7.30 for initial cytotoxicity, for plate incorporation and preincubation methods to get the concentration of 10% (v/v).

4.4.4. Positive controls

For cultures with metabolic activation, 2-aminoanthracene was used for strains TA98, TA100, TA1535, TA1537 at a concentration of 4.0 µg/plate, and for strain WP2

uvrA (pKM 101) at a concentration of 30.0 µg/plate. For cultures without metabolic activation, 2-nitrofluorene was used for strain TA98 at a concentration of 2.0 µg/plate, sodium azide was used for strains TA100 and TA1535 at a concentration of 1.0 µg/plate, 9-aminoacridine at a concentration of 50 µg/plate was used for strain TA1537, and 4-nitroquinoline N-oxide was used for strain WP2 uvrA (pKM 101) at a concentration of 5.0 µg/plate. All positive controls were dissolved in DMSO.

4.4.5. Solubility, precipitation, and cytotoxicity tests

In order to determine the appropriate vehicle and concentration range for the main test, the following preliminary tests were performed. The solubility test was carried out with water and DMSO at 50 mg/ml. The precipitation test was conducted with different concentrations of 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 5 mg/plate, mixing the test compound dissolved in DMSO with molten soft agar, and incubated for 2 hours at 37±1ºC. For the cytotoxicity test, the same concentrations described above were added to a bacterial culture of *S. typhimurium* TA100 in triplicate, both in the presence and absence of metabolic activation along with concurrent vehicle control (DMSO). Each concentration of test item was mixed with soft agar containing histidine and biotin, S9 mix (for presence of metabolic activation), Phosphate Buffer Saline (for absence of metabolic activation), *Salmonella typhimurium* TA100 of cell density approximately 18×10^8 cells/mL overlaid on to pre-labeled minimal glucose agar plates. The plates were incubated at 37±1ºC for 48 hours and 30 minutes.

4.4.6. Plate incorporation method

Plate incorporation method was carried out with test concentrations 0.05, 0.16, 0.5, 1.6 and 5 mg/plate of test item, vehicle and positive control. These five concentrations of the test item were plated, with each of the following tester strains: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101) with and without metabolic activation. Each culture was conducted in triplicate.

The tester strains (0.1 ml) were mixed with 0.5 ml of S9 mix or phosphate buffer saline and 2 ml molten soft agar containing histidine-biotin/tryptophan and poured on to minimal glucose agar plates. Then, 0.1 ml of the test article solution, vehicle control, or positive control were added. Plates were incubated at 37±1°C for 48 hours and 5

minutes. The condition of the bacterial background lawn was evaluated for evidence of the test item cytotoxicity using the code system and revertant colonies for each strain within the test item dilution series were counted manually.

4.4.7. Preincubation method

Preincubation method was carried out with concentrations of 0.05, 0.16, 0.5, 1.6 and 5 mg/plate of the test item, vehicle and positive control. The tester strains (0.1 ml) along with S9/Phosphate Buffer Saline (0.5 ml) were transferred into sterile test tubes and incubated in an incubator shaker for 21 to 23 minutes at 37±1°C at 100±5 g. Post incubation, the test constituents were mixed with 2 ml molten soft agar containing histidine-biotin for *Salmonella typhimurium* and L-tryptophan for *E. coli* WP2 uvrA (pKM101) and poured on to minimal glucose agar plates. Five concentrations of the test item, vehicle or positive control (0.1 ml) were plated, with each of the following tester strains: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101) with and without metabolic activation. Plates were incubated at 37±1°C for 49 hours and 24 minutes.

The condition of the bacterial background lawn was evaluated for evidence of the test item cytotoxicity using the code system and revertant colonies for each strain within the test item dilution series were counted manually.

4.4.8. Evaluation of results

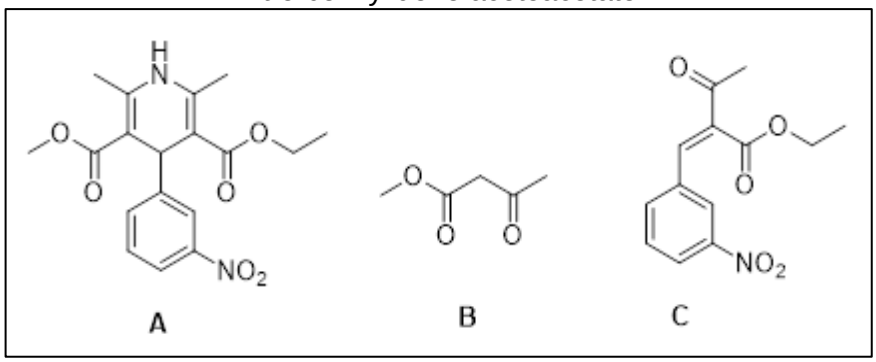
The mean and standard deviation of the number of revertants per plate were calculated. If a 2-fold or more increase in the number of revertant colonies to that of spontaneous revertant colonies (the vehicle control) were noted, the test article was considered to be positive for mutagenicity. The dose-response relationship was also evaluated. The obtained result was then compared to the mutagenicity prediction given by the *in silico* systems.

4.5 ANALYTICAL PROCEDURE DEVELOPMENT FOR NITRENDIPINE IMPURITIES

An analytical method was developed with the aim to evaluate the exposure during the risk assessment of two potentially mutagenic impurities arising from the drug substance nitrendipine. The impurities are named methyl acetoacetate and ethyl-3-nitro benzylidene acetoacetate, and their structures as well as the structure of nitrendipine are presented in Figure 2.

The mutagenicity prediction was performed for these impurities using the settings as described in Table 5, and the acceptable limit was calculated to establish the sensitivity required for the method. The limit was calculated to be 37.5 ppm for each impurity, considering the TTC (1.5 µg/day) and the maximum daily dose of nitrendipine as described in the drug product leaflet (40 mg/day). The desired sensitivity for the method was a limit of detection of at least 11.25 ppm, corresponding to 30% of the acceptable limit.

Figure 2. Chemical structures of (**A**) nitrendipine, (**B**): methyl acetoacetate and (**C**): ethyl-3-nitro benzylidene acetoacetate



4.5.1 Materials

The drug substance nitrendipine was obtained from Uquifa S.A. (Union Quimico Farmaceutica S.A.). The standards of impurities were acquired from Axios Research.

4.5.2 Equipment

For separation and detection of the impurities, the UHPLC equipment used was an Agilent 1290 Infinity, coupled to a Q-TOF mass spectrometer model 6540, with a MassHunter Workstation software (Agilent Technologies); for sample preparation, the equipments used were a Heidolf shaker, a Hettich centrifuge, and a Metler Toledo analytical balance.

4.5.3 Sample and standard preparation

The sample was prepared by adding 1 g of drug substance to a 50 ml falcon tube and dissolved in 5 ml ultrapurified water. The sample was shaked during 10 min in maximum velocity and centrifuged for 15 min at 9000 g under room temperature. 90 µl of the supernatant was added to an insert for the analysis.

A stock solution containing the impurities was prepared at a concentration of 500 ppm by dissolving 10 mg of each impurity in 20 ml acetonitrile. This solution was diluted to a 125 ppm solution by taking 1.25 ml of the stock solution and completing to volume with acetonitrile in a 5 ml volumetric flask. Finally, 90 µl of the 125 ppm solution was added to a 5 ml volumetric flask and completed to volume with ultrapurified water. The concentration of the final solution was 2.25 ppm, corresponding to 11.25 ppm with reference to the sample concentration. 90 µl of this solution was added to an insert for the analysis.

A spiked sample was prepared by adding 90 µl of the 125 ppm standard solution to 1 g of sample and completing with ultrapurified water to achieve a final volume of 5 ml. This spiked sample was shaked during 10 min in maximum velocity and centrifuged for 15 min at 9000 g under room temperature. Ninety µl of the supernatant was added to an insert for the analysis.

4.5.4 Chromatographic and mass spectrometer conditions

UHPLC analysis was performed using an Agilent UHPLC/Q-TOF-MS system (Agilent, USA), using the conditions described in Table 6.

Table 6. Chromatographic conditions

Parameter	Condition
Injection volume	20 µl
Column	ACQUITY UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm, Waters)
Column temperature	30°C
Flow rate	0.4 ml/min

The UHPLC separation system included a binary solvent system with mobile phase A (0.1% formic acid in 10mM ammonium bicarbonate solution) and mobile phase B (0.1% formic acid in acetonitrile). The gradient used is described in Table 7.

Table 7. Mobile phase gradient

Time (min)	Mobile phase A (%)	Mobile phase B (%)
3	5	95
5	60	40
9	30	70
11	15	85
12	30	70
13	30	70
13.1	97	3
15	97	3

The retention time was 2.57 minutes for methyl acetoacetate and 8.39 minutes for ethyl-3-nitro benzylidene acetoacetate.

Q-TOF-MS was equipped with electrospray ionisation in positive mode. The MS parameters are described in Table 8.

Table 8. Mass spectrometer conditions

Parameter	Condition
Drying gas temperature	350°C
Drying gas flow	8 l/min
Nebulizer pressure	35 psig
Sheath gas temperature	350°C
Sheath gas flow	11 l/min
Capillary voltage	4 kV
Nozzle voltage	2 kV
Fragmentor voltage	90 V
Skimmer voltage	46 V
Octapole voltage	750 V
Evaporative and auxiliary gas	High purity nitrogen
Reference ions	$[M + H]^+$ 121.050873 and 922.009798
Range of data acquisition	100-950 *m/z*

4.5.5 Optimization of detection method by Q-TOF-MS

The ionization method was first optimized for the drug substance, since it shows a degree of structural similarity with one of the impurities. MS settings were set by direct infusion of a 10 ppm nitrendipine solution. Mobile phase composition, flow rate, drying gas flow and temperature, nebulizer flow and Sheath gas flow and temperature are interdependent parameters, and were optimized to increase analytes desolvatation, enabling a greater amount of species to reach the detector. Capillary and Nozzle voltage were set considering low pH of the mobile phase, increasing protonation and consequently, sensitivity of the method. Finally, Fragmentor and Skimmer potentials were set to provide the higher energy possible, without source fragmentation. After having the ionization source parameter optimized based on the

drug substance, potentials were slightly adjusted to allow good sensitivity of the impurities.

4.5.6 Validation of the analytical method

The analytical validation was performed following the requirements described in the RDC 166/2017, the current regulatory document that sets validation criteria for pharmaceuticals in Brazil. Since in this case the aim of the procedure was not to quantify the impurities, but rather to assess whether they were above or below the acceptable limit, a limit test validation was performed. The required parameters for such a validation are specificity and limit of detection, so the parameters that would be necessary for quantification (accuracy, precision, linearity) are not required, neither by RDC 166/2017, nor by the international guideline for analytical validations, ICH Q2.

4.5.6.1 Specificity

The specificity of the method was assessed by injecting a sample spiked with the impurities and the standard solution of impurities. The retention time in the chromatographic run as well as the exact mass in the Q-TOF-MS should match for each impurity.

4.5.6.2 Limit of detection

The desired limit of detection was based on the concentration corresponding to 30% of the acceptable limit for the impurities in the drug substance. The sample was spiked with the impurities at this level to confirm that the method would have the adequate sensitivity. The signal to noise ratio should be higher than 3 for each impurity.

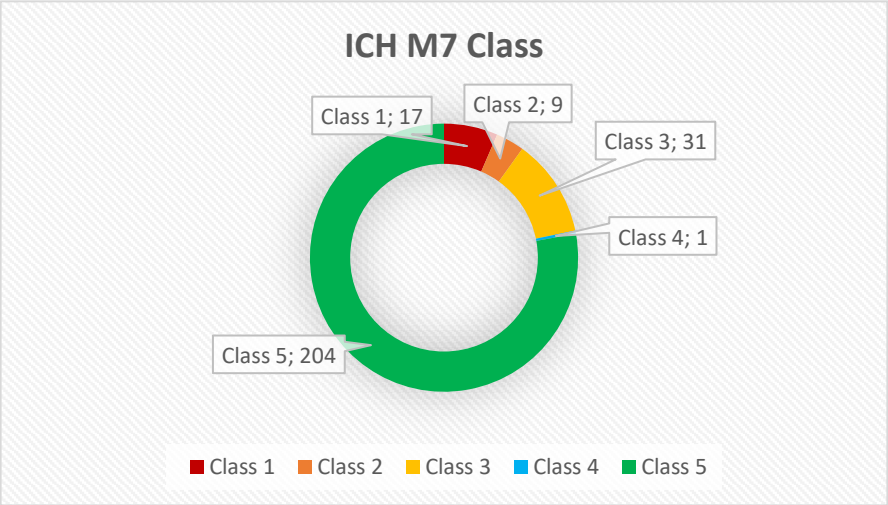
5 RESULTS

5.1 MUTAGENICITY ASSESSMENT

A total of 262 impurities have been identified based on the information presented in the drug master file, and had their mutagenic potential predicted through the *in silico* systems – Derek and Sarah Nexus, associated with the critical evaluation after the prediction – the expert review. As a result of the prediction after the expert review, each impurity was classified in one of the five classes defined in ICH M7.

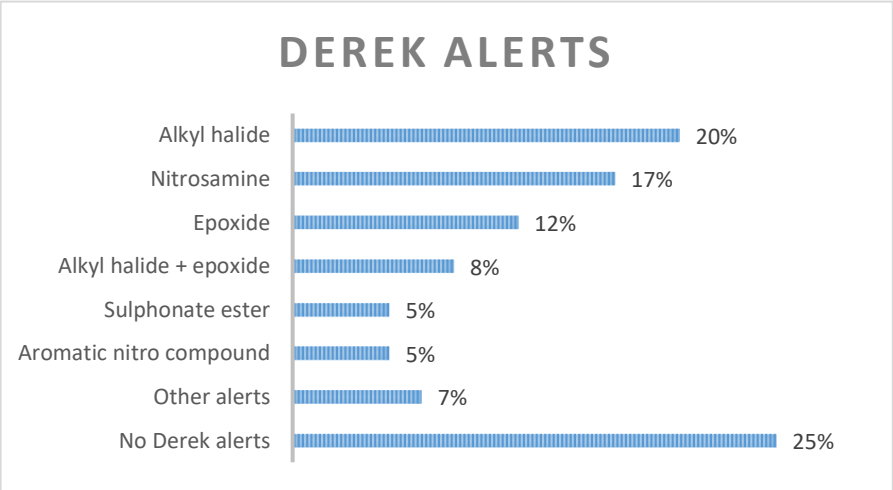
Figure 3 summarizes the results of this classification. The majority (78%) of impurities were class 5, hence considered as not of concern regarding mutagenicity. Only one impurity was assigned to class 4, which means it shows a mutagenicity alert however this alert is shared with another compound which presents negative mutagenicity data. Hence class 4 impurities are also considered as not of concern regarding mutagenicity. The remaining 22% impurities were classified as potentially mutagenic – classes 1, 2 and 3.

Figure 3. Classification of impurities regarding their mutagenic potential.



The main Derek alerts fired for class 1, 2 and 3 impurities are presented in Figure 4. The most common structural alerts for mutagenicity in impurities arising from anti-hypertensive drug substances were found to be related to alkyl halides, nitrosamines and epoxides.

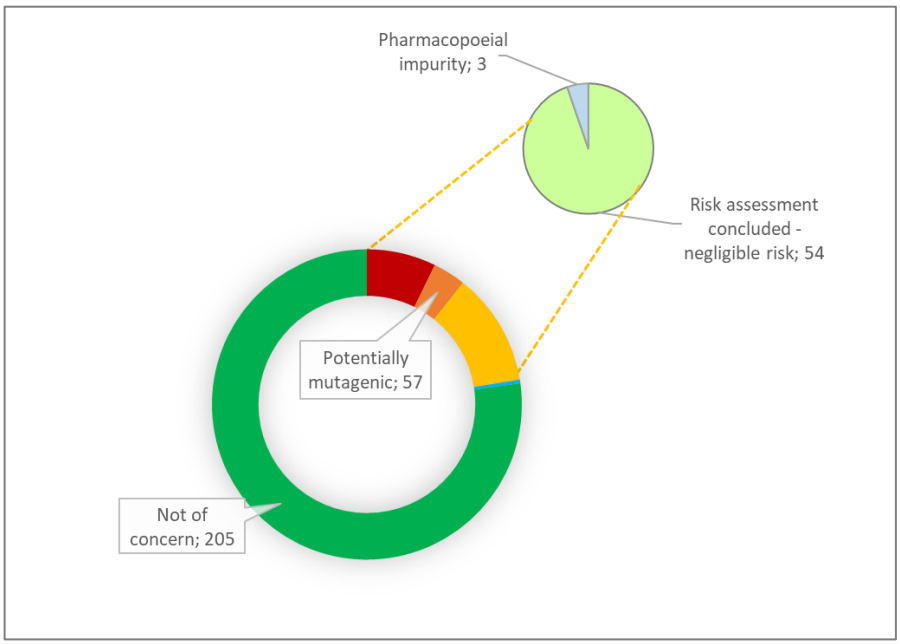
Figure 4. Derek alerts fired for potentially mutagenic impurities.



5.2 RISK ASSESSMENT

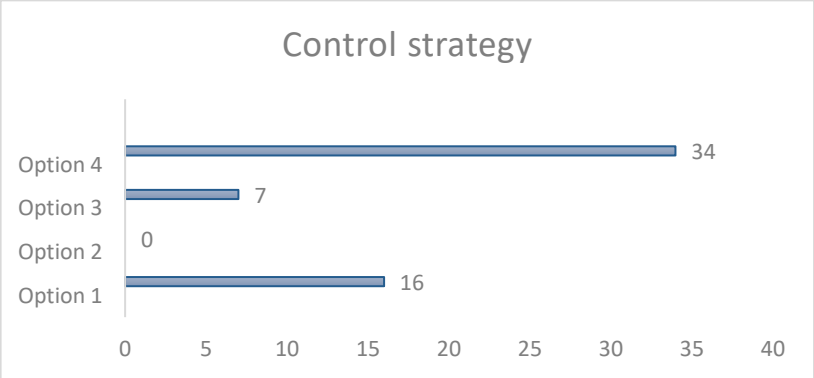
For each of the 57 potentially mutagenic impurities identified, the risk assessment was performed. For 54 impurities, the levels of the impurity were found to be below the acceptable limit. This indicates that these impurities do not pose a risk higher than the negligible risk considered acceptable according to ICH M7. The other 3 impurities are described in a pharmacopoeial monograph, so although they presented a mutagenicity alert, the limits established by the manufacturers were based on the pharmacopoeia. Figure 5 shows a summary of the risk assessment results.

Figure 5. Risk assessment results



After concluding the risk assessment, as recommended by ICH M7, a control strategy must be defined for each potentially mutagenic impurity. The control strategies adopted for the impurities are described in Figure 6. For 34 impurities (60%), option 4 was established, which is based on the purge factor. For 16 impurities (28%), option 1 was chosen, which consisted of a decision to include a test for the impurity in the drug substance specification – either as routine or periodic testing. For 7 impurities (12%), option 3 was adopted, which consists of establishing a test for the impurity in an intermediate stage, with a limit higher than the limit that would be acceptable in the drug substance. No manufacturer chose to adopt option 2.

Figure 6. Control strategies for the impurities



5.3 THE AMES TEST FOR ATENOLOL IMPURITY D

5.3.1. Solubility, precipitation and cytotoxicity tests

Atenolol impurity D was found soluble in dimethyl sulphoxide at a concentration of 50 mg/ml and resulted in no precipitation from 0.00625 to 5 mg/plate at the tested concentrations. It also resulted in no cytotoxicity from 0.00625 to 5 mg/plate with lawn intensity (4+) thick lawn in the presence and absence of metabolic activation system when compared to vehicle control 4+ (Thick lawn).

5.3.2. Plate incorporation and preincubation methods

The results are presented in Tables 9 and 10. In the two trials conducted, impurity D resulted in no appreciable increase in the number of revertant colonies over the vehicle control in *Salmonella typhimurium* TA98 and *E. coli* WP2 uvrA (pKM101)

tester strains. However for both trials, it did result in slight increase in the number of revertant colonies over the vehicle control in TA100 (up to 1.7 fold increase, only for the highest tested concentration) and appreciable increase in the number of revertant colonies over the vehicle control in TA1535 and TA1537, with and without S9 (from 6.1 to 36.8 fold increase across the tested concentrations). The positive controls tested simultaneously resulted in 3.8 to 14.9-fold increase in the number of revertant colonies/plate under identical conditions, confirming the assay validity. The dose-response relationship for these two strains TA1535 and TA1537 is demonstrated in Figure 7.

These results provide experimental evidence that impurity D is mutagenic in bacteria, hence although originally being assigned to class 3, after the results of the Ames test this impurity was assigned to class 2 as per ICH M7 classification.

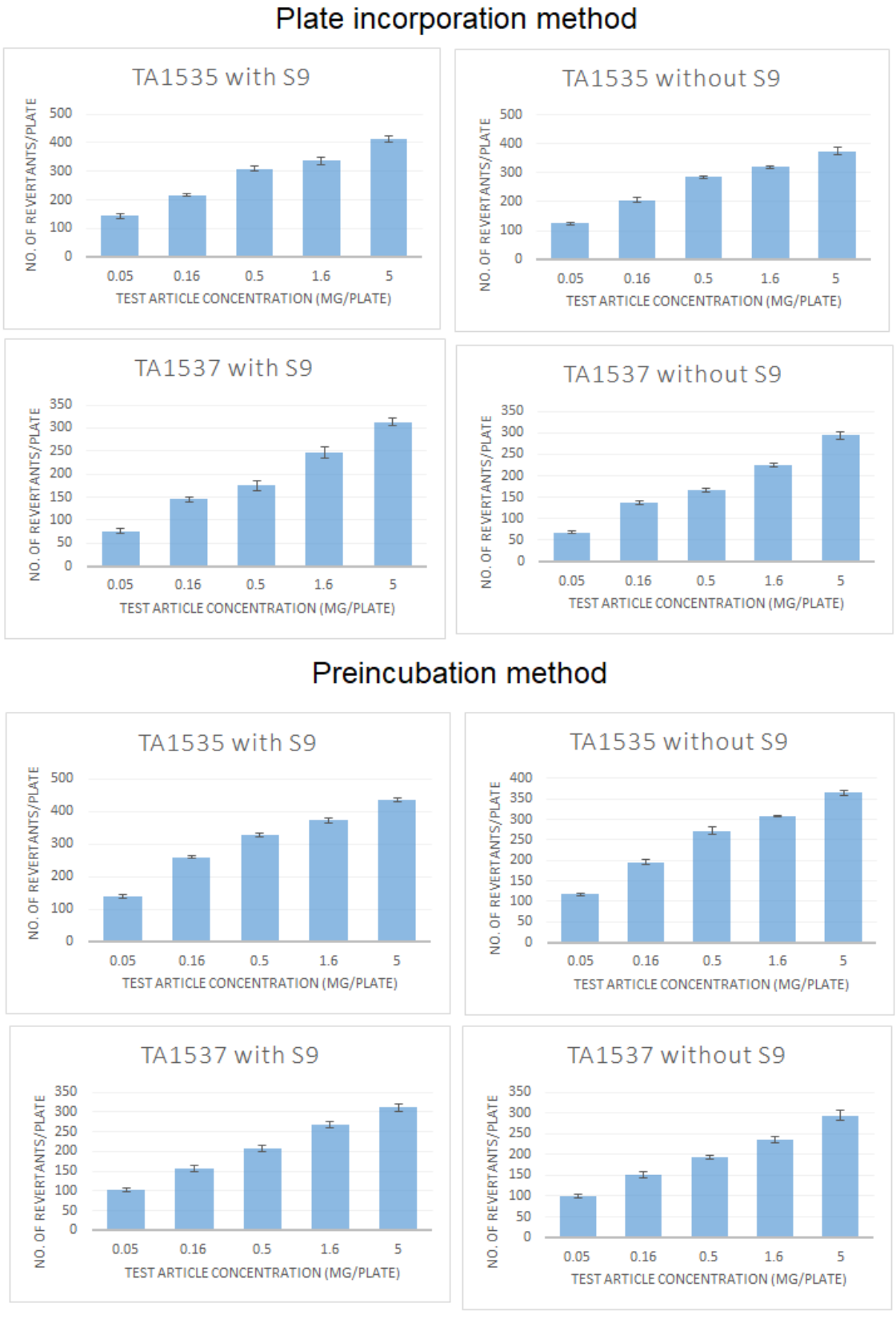
Table 9. Mutagenicity test results – Plate incorporation method

With or without S9	**Test article concentration (mg/plate)**	**Mean No. of Revertants/Plate (± SD)**				
		TA98	TA100	TA1535	TA1537	WP2uvrA (pKM101)
With S9	Vehicle control	28.3 ± 2.1	101.3 ± 3.1	19.7 ± 2.5	10.7 ± 1.5	74.3 ± 2.5
	Positive control	385.0 ± 9.6	405.7 ± 4.5	144.0 ± 5.0	122.0 ± 5.6	386.7 ± 11.1
	0.05	27.7 ± 3.1	96.7 ± 3.1	143.7 ± 8.5	76.0 ± 5.6	70.3 ± 2.5
	0.16	26.3 ± 2.1	96.7 ± 4.5	216.3 ± 4.0	145.0 ± 4.6	73.7 ± 3.5
	0.5	29.0 ± 2.0	98.3 ± 5.9	308.3 ± 9.6	176.0 ± 10.8	70.7 ± 1.5
	1.6	28.3 ± 2.1	97.7 ± 4.0	336.3 ± 12.7	246.7 ± 11.7	72.0 ± 3.6
	5	29.0 ± 2.0	168.0 ± 7.0	411.3 ± 11.9	313.0 ± 8.2	72.3 ± 5.7
Without S9	Vehicle control	24.7 ± 2.1	97.0 ± 2.6	20.0 ± 1.0	8.7 ± 1.5	71.0 ± 2.0
	Positive control	364.3 ± 10.6	389.0 ± 5.6	139.7 ± 3.5	119.7 ± 4.5	376.3 ± 11.9
	0.05	24.3 ± 2.1	95.3 ± 5.9	125.0 ± 4.6	68.7 ± 2.5	70.0 ± 4.0
	0.16	24.0 ± 3.0	96.0 ± 2.6	205.0 ± 8.5	136.63 ± 4.7	69.0 ± 3.6
	0.5	25.0 ± 3.0	95.7 ± 5.5	284.3 ± 5.5	166.0 ± 4.0	71.3 ± 3.1
	1.6	24.3 ± 2.5	95.7 ± 5.5	319.3 ± 4.5	225.0 ± 4.6	70.3 ± 3.5
	5	24.7 ± 1.5	158.3 ± 6.0	374.7 ± 11.9	294.0 ± 8.2	70.3 ± 2.1

Table 10. Mutagenicity test results – Preincubation method

With or without S9	Test article concentration (mg/plate)	Mean No. of Revertants/Plate (± SD)				
		TA98	TA100	TA1535	TA1537	WP2uvrA (pKM101)
With S9	Vehicle control	28.7 ± 2.1	103.0 ± 7.0	20.7 ± 2.5	11.7 ± 1.5	77.3 ± 3.8
	Positive control	387.0 ± 14.0	402.3 ± 11.6	145.0 ± 9.0	124.7 ± 5.1	383.0 ± 12.0
	0.05	28.0 ± 2.0	97.0 ± 6.2	138.3 ± 6.5	101.7 ± 4.5	71.3 ± 2.5
	0.16	27.7 ± 3.1	98.7 ± 4.7	260.3 ± 4.0	156.0 ± 7.5	71.7 ± 3.5
	0.5	26.7 ± 1.2	102.0 ± 4.0	327.0 ± 6.6	206.7 ± 8.1	71.0 ± 3.0
	1.6	27.3 ± 2.5	106.7 ± 4.2	373.3 ± 7.0	267.7 ± 6.7	74.3 ± 1.5
	5	27.7 ± 1.5	169.3 ± 4.0	435.0 ± 5.6	311.0 ± 9.5	78.3 ± 2.5
Without S9	Vehicle control	25.0 ± 1.0	102.0 ± 3.6	19.3 ± 1.5	8.0 ± 1.0	72.0 ± 3.0
	Positive control	372.3 ± 4.2	390.7 ± 7.5	140.7 ± 8.6	117.7 ± 5.5	381.3 ± 11.4
	0.05	24.0 ± 2.6	99.7 ± 2.1	117.0 ± 3.6	100.3 ± 4.0	70.3 ± 2.5
	0.16	23.0 ± 2.6	96.7 ± 4.0	195.3 ± 5.5	151.7 ± 7.5	68.0 ± 2.0
	0.5	23.0 ± 2.6	98.3 ± 5.1	271.3 ± 9.1	192.7 ± 4.5	70.0 ± 2.0
	1.6	23.3 ± 1.5	98.0 ± 3.0	307.7 ± 2.5	235.3 ± 8.5	70.0 ± 1.0
	5	24.0 ± 2.0	158.7 ± 4.0	365.0 ± 6.2	294.0 ± 11.8	70.3 ± 1.5

Figure 7. Dose-response relationship for impurity D in strains TA1535 and TA1537, with and without S9, using plate-incorporation and preincubation methods.



5.4 ANALYTICAL PROCEDURE DEVELOPMENT FOR NITRENDIPINE IMPURITIES

As part of the exposure evaluation for two impurities from nitrendipine, an analytical method was developed to determine their level in the drug substance, since the purge ratio was not high enough to justify their elimination without additional data.

5.4.1 Mutagenicity prediction of impurities and expert review

The results of the mutagenicity prediction for the nitrendipine impurities are presented in Table 11. Both impurities were classified as potentially mutagenic (classes 2 or 3).

Table 11. Summary of classification of nitrendipine impurities.

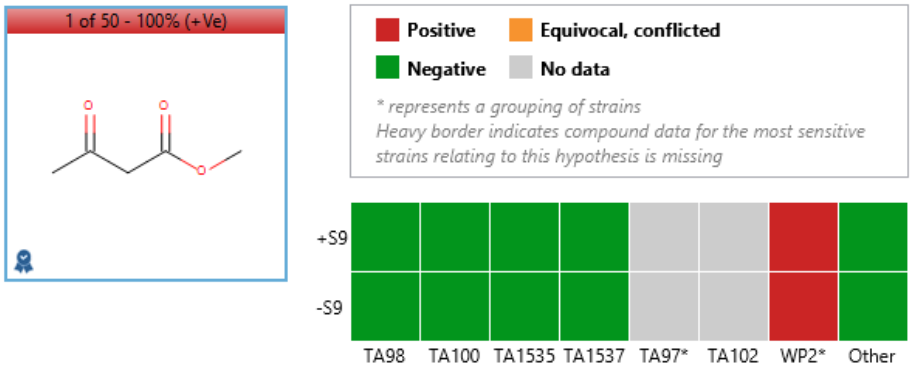
Impurity	CAS number	Derek Prediction	Sarah Prediction	Similarity to API*	Overall Carcinogenicity	Overall Ames	ICH M7 Class
Methyl acetoacetate	105-45-3	Inactive: No misclassified or unclassified features	Positive – 100%	No Derek Alerts found	Unspecified	Active	2
Ethyl-3-nitro benzylidene acetoacetate	39562-16-8	Plausible: Alert329 – Aromatic nitro compound	Equivocal	Alert(s) not found in API	Unspecified	Unspecified	3

*API = Active pharmaceutical ingredient (drug substance)

Methyl acetoacetate was classified as class 2, indicating that a mutagenicity study is available for this compound, with a positive outcome. The evaluation of the strain information presented by the Sarah Nexus interface (Figure 8) indicates that the positive result for this compound was only for the *E. coli* WP2 strain. Nonetheless, other databases such as the ECHA website have found this compound to be non-mutagenic (ECHA, 2021). Other available studies which have been appropriately conducted under GLP and according to OECD, including the use of the WP2 strain, have also found negative results (JAPAN NATIONAL INSTITUTE OF HEALTH SCIENCES, 2021; SHIMIZU et al., 1985). This difference in results can be explained by the fact that more than one WP2 strain can be used in the Ames test - *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101) (OECD, 2020). The pKM101 is a strain which has been added a plasmid which increases the mutagen sensitivity (WILLIAMS et al.,

2019). While the studies presenting negative results have used the less sensitive strain (without pKM101), this may be the reason for the differences when compared to Lhasa database, where the positive result relates to a study using the more sensitive strain. Hence, the impurity was classified as class 2.

Figure 8. Strain information presented by Sarah Nexus for methyl acetoacetate



Ethyl-3-nitro benzylidene acetoacetate is a class 3 impurity since it is an aromatic nitro compound. Based on the information provided by Derek Nexus, the mutagenicity of this class of compounds begins with their reduction by nitroreductases to the hydroxylamine, and then they undergo *O*-esterification, generating a product which may give rise to a nitrenium ion, which is the electrophile ultimately reacting with DNA.

Considering that nitrendipine itself is an aromatic nitro compound, at first a possible classification into class 4 could be proposed, since the drug substance is not expected to be mutagenic, although it presents the same mutagenicity structural feature as the impurity. However, when the mutagenicity prediction is done for nitrendipine in Derek Nexus, no alert for mutagenicity is fired. This means that the software does not consider this compound to be within the rules described by the aromatic nitro alert.

When looking even deeper at the information provided by Derek, some exceptions to the rule are described. One of the exceptions are “nitrobenzenes substituted in the 2- or 3- position with sterically demanding dihydropyridines”. The examples presented include nifedipine, which is a drug substance structurally similar to nitrendipine, differing by a methyl group and by the position of the dihydropyridines, which is in the 2- position for nifedipine, but in the 3- position for nitrendipine. Hence,

such compounds are not mutagenic, probably due to the steric hindrance caused by the dihydropyridine ring. Nonetheless, the impurity ethyl-3-nitro benzylidene acetoacetate does not contain such a ring, so it does not fit into the exceptions of the aromatic nitro mutagenicity alert. This explains why the alert was fired by Derek for this compound, and why it should not be considered a class 4 impurity.

Impurities can only be assigned to class 4 when the mutagenicity alert is the same present in a non-mutagenic compound, containing the same chemical structural environment. In this case, the structural similarity cannot be established, since an important feature which removes the mutagenicity concern is present in the drug substance but not in the impurity. In conclusion, ethyl-3-nitro benzylidene acetoacetate has been classified as a class 3 impurity.

Before proceeding to the analytical method development, the purge factor was calculated for both impurities, as an attempt to verify their potential elimination from the process. However, since they are relatively close to the drug substance in the synthesis, meaning that there are few chemical transformation steps separating them from nitrendipine, the purge ratio was found to be less than 100, although it was higher than 1. Hence, additional experimental data should be provided to support the result, and a suitable analytical procedure was developed for this purpose.

5.4.2 Analytical validation results

5.4.2.1 Specificity

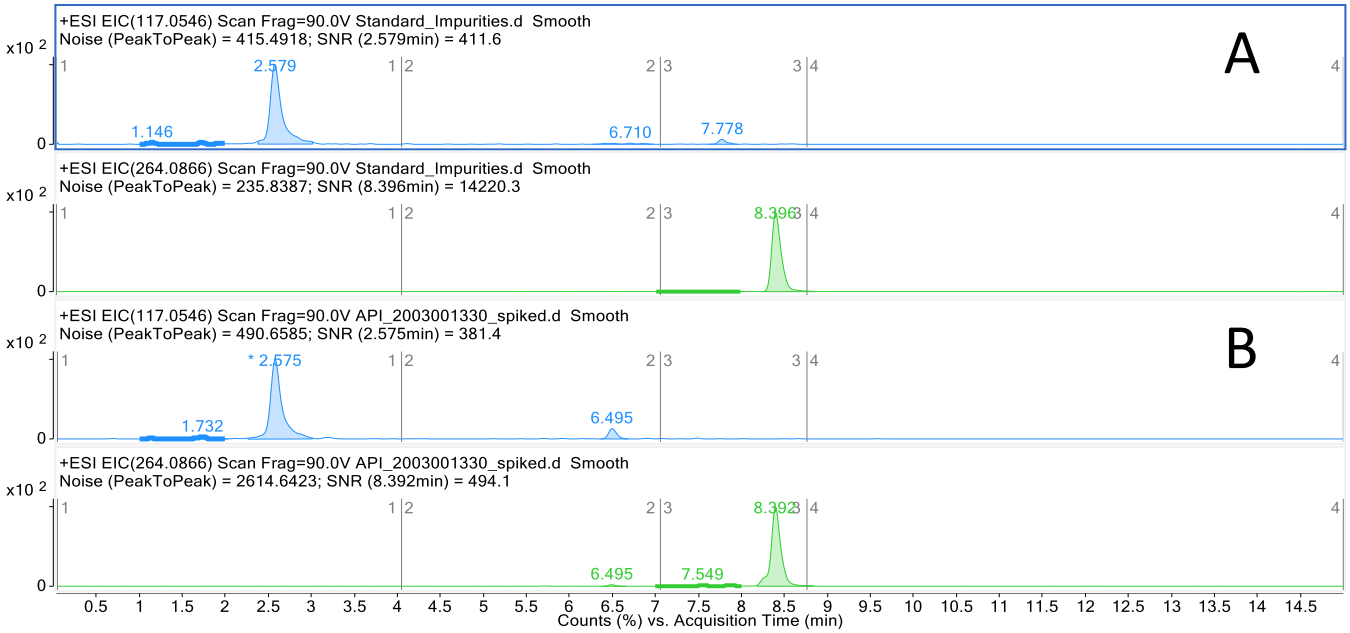
The retention time of impurities in the standard solution was compared to their retention time in the spiked sample solution. The results are summarized in Table 12. Both impurities are well separated from each other and from the drug substance nitrendipine. The specificity chromatograms corresponding to the standard solution and spiked sample solution are presented in Figure 9.

Table 12. Retention time of the impurities in standard solution and spiked samples

Compound	Standard solution	2003000275 spiked	2003000276 spiked	2003001330 spiked
Methyl acetoacetate	2.579 min	2.562 min	2.562 min	2.575 min
Ethyl-3-nitro benzylidene acetoacetate	8.396 min	8.393 min	8.392 min	8.392 min

Besides the comparison of the retention time, the second criteria indicating specificity of the method was the mass spectrum. The spectra corresponding to methyl acetoacetate in the standard solution and in the spiked sample solution are provided in Figure 10, and for ethyl-3-nitro benzylidene acetoacetate the spectra are presented in Figure 11.

Figure 9. Chromatogram of (**A**) standard solution and (**B**) spiked sample solution, indicating similarity in the retention times of methyl acetoacetate (2.57 min) and ethyl-3-nitro benzylidene acetoacetate (8.39 min).



For both impurities, the ions detected by the method correspond to their protonated form $[M + H]^+$, 117.0546 *m/z* for methyl acetoacetate, and 264.0866 *m/z* for ethyl-3-nitro benzylidene acetoacetate. Since these signals were found at their respective retention times in the spectra of the standard and spiked sample, this confirms the identity of the impurities and the specificity of the method for both.

Figure 10. Mass spectra of methyl acetoacetate in (**A**) standard solution and (**B**) spiked sample solution.

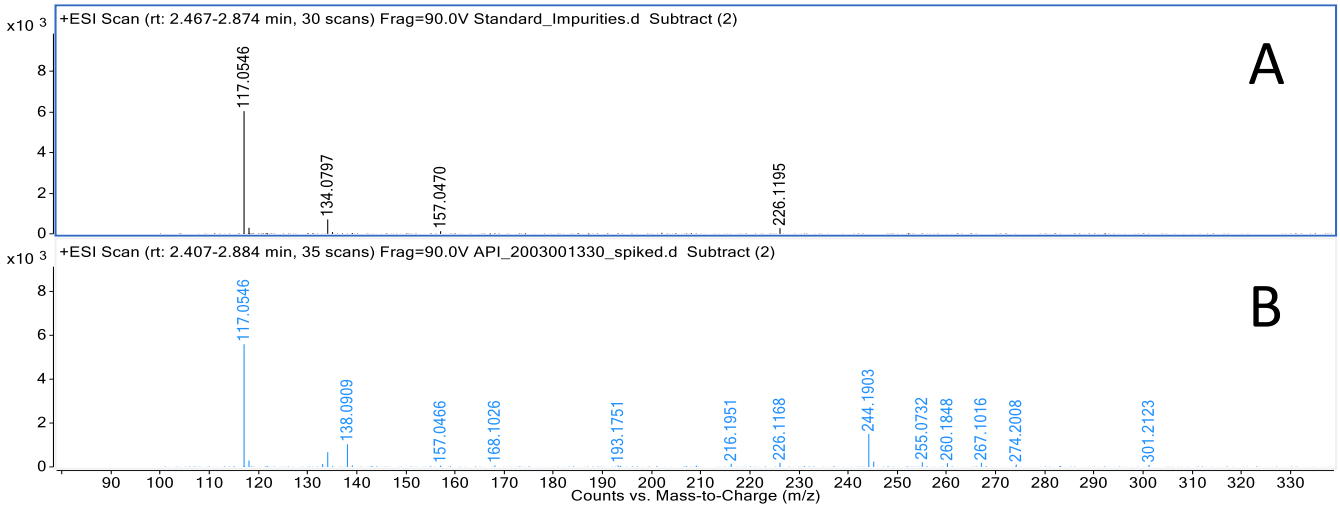
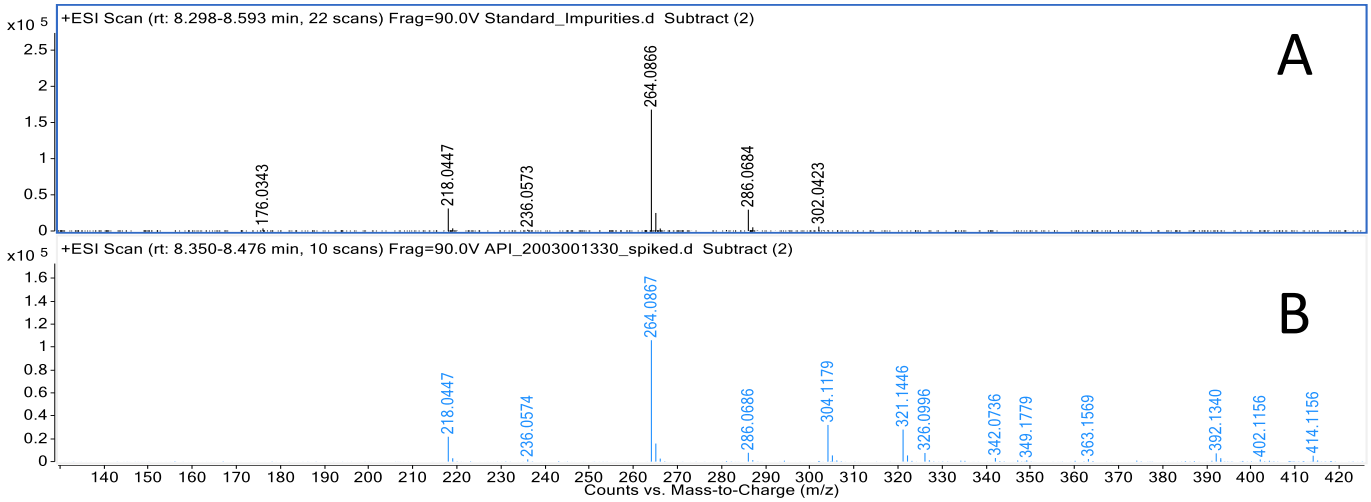


Figure 11. Mass spectra of ethyl-3-nitro benzylidene acetoacetate in (**A**) standard solution and (**B**) spiked sample solution.



5.4.2.2 Limit of detection

The signal to noise (S/N) ratio for each impurity at the concentration of 11.25 ppm with reference to sample is presented in Table 13.

Table 13. Signal to noise ratio at 11.25 ppm

Impurity	2003000275 spiked	2003000276 spiked	2003001330 spiked
Methyl acetoacetate	292.6	281.4	381.4
Ethyl-3-nitro benzylidene acetoacetate	564.3	559.9	494.0

Since the signal to noise ratio is higher than 3 for both impurities, they meet the requirement for establishing an appropriate limit of detection for this method. Considering that the S/N ratio is approximately 100 times higher than the requirement, this indicates that a significantly lower detection limit could be achieved, probably in the range of ppb. Nonetheless, the target of this method was 30% of the acceptable limit for the impurities, so lower concentrations were not prepared.

5.4.4 Level of impurities in the drug substance

The results found for each impurity in three batches of the drug substance are presented in Table 14.

Table 14. Experimental results of nitrendipine impurities in 3 batches of the drug substance

Impurity	Nitrendipine batch number		
	2003000275	2003000276	2003001330
Methyl acetoacetate	BDL	BDL	BDL
Ethyl-3-nitro benzylidene acetoacetate	BDL	BDL	BDL

*BDL = Below detection limit

For all tested batches, the levels of the impurities were below the detection limit, confirming that they are eliminated to less than 30% of their acceptable limit in the drug substance. Hence, the risk assessment was concluded confirming a negligible risk.

5.5 COMPARISON OF *IN SILICO* VS. EXPERIMENTAL RESULTS

5.5.1 The purge factor vs. analytical results for a nitrosamine

The purge factor calculation was compared to experimental results for a nitrosamine impurity in a ‘sartan’ drug substance. The formation of nitrosamines in these routes of synthesis are usually related to the presence of a secondary amine and nitrous acid, which react in certain conditions yielding the corresponding nitrosamine impurity (ROSTKOWSKA et al., 1998).

This was an issue with ‘sartans’ especially because their structures contain the tetrazole moiety, which is formed through the reaction of sodium azide with a nitrile group. Sodium azide may contain sodium nitrite as a contaminant, which may originate in its synthesis. Moreover, quenching of azide may be performed using sodium nitrite

itself in the manufacturing process of the drug substance. The introduction of sodium nitrite to the manufacturing process, along with an acidic medium, provides nitrous acid (HOLLEMAN, A. F.; WIBERG, E., WIBERG, 2001; JIA YULIANG ZHANG FUCHENG, 2015).

Secondary amines, on the other hand, may be contaminants of organic solvents which are commonly used in the manufacturing process of drug substances, such as dimethylformamide, triethylamine, N-methylpyrrolidone, and N,N-diisopropylethylamine. They can also be found in the structure of starting materials, intermediates or the drug substance itself. The stage of the synthesis when these amines encounter nitrous acid is where the nitrosamine can be formed. In the case of 'sartans', this is usually the stage when the tetrazole moiety is obtained (EMA, 2019d).

If formation of the tetrazole occurs in a late step of the synthesis – only a few stages before obtaining the final drug substance, there is a higher chance that the level of the impurity will exceed the acceptable limit. On the other hand, when this step is performed early in the synthesis, there are plenty of stages and opportunity to eliminate and purge the impurity before obtaining the final drug substance.

This was the case presented here – Case 1 in section 5.6.1. For this drug substance, a certain route of synthesis was used, where formation of the tetrazole moiety was done during the synthesis of the starting material – that is, many stages away from the final drug substance. The purge factor calculation showed that the expected result for this impurity was 4×10^{-14} ppm, hence allowed for option 4 to be adopted for this nitrosamine (purge ratio was >1000).

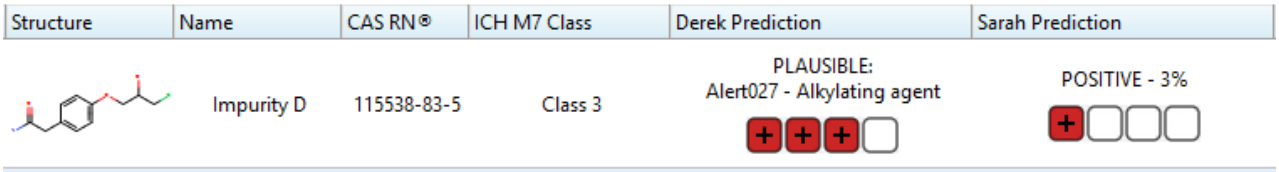
In order to confirm the result of the prediction, the drug substance manufacturer presented analytical results of the impurity in the drug substance using a validated method with a limit of detection of 0.008 ppm. The impurity was not detected in any of the 25 batches tested, hence confirming its elimination. The analytical method used one of the most sensitive approaches currently available – LC-MS – and yet was not close to the estimated result predicted by the purge factor calculation. Unfortunately, making a clear direct comparison between predicted and measured purge may not be possible in many cases due to analytical limitations (BURNS et al., 2019).

However, both results prove that the impurity was in fact eliminated and had its levels less than 0.008 ppm, which is well below the acceptable limit in this case. Hence, the purge factor was able to predict the analytical result.

5.5.2 Mutagenicity prediction vs. the Ames test for atenolol impurity D

The Ames test result was compared to the mutagenicity prediction for the compound 2-[4-[(2RS)-3-chloro-2-hydroxypropoxy] phenyl]-acetamide (Atenolol impurity D). The *in silico* prediction for bacterial mutagenicity yielded a plausible prediction by Derek and a positive prediction by Sarah, as shown in Figure 12.

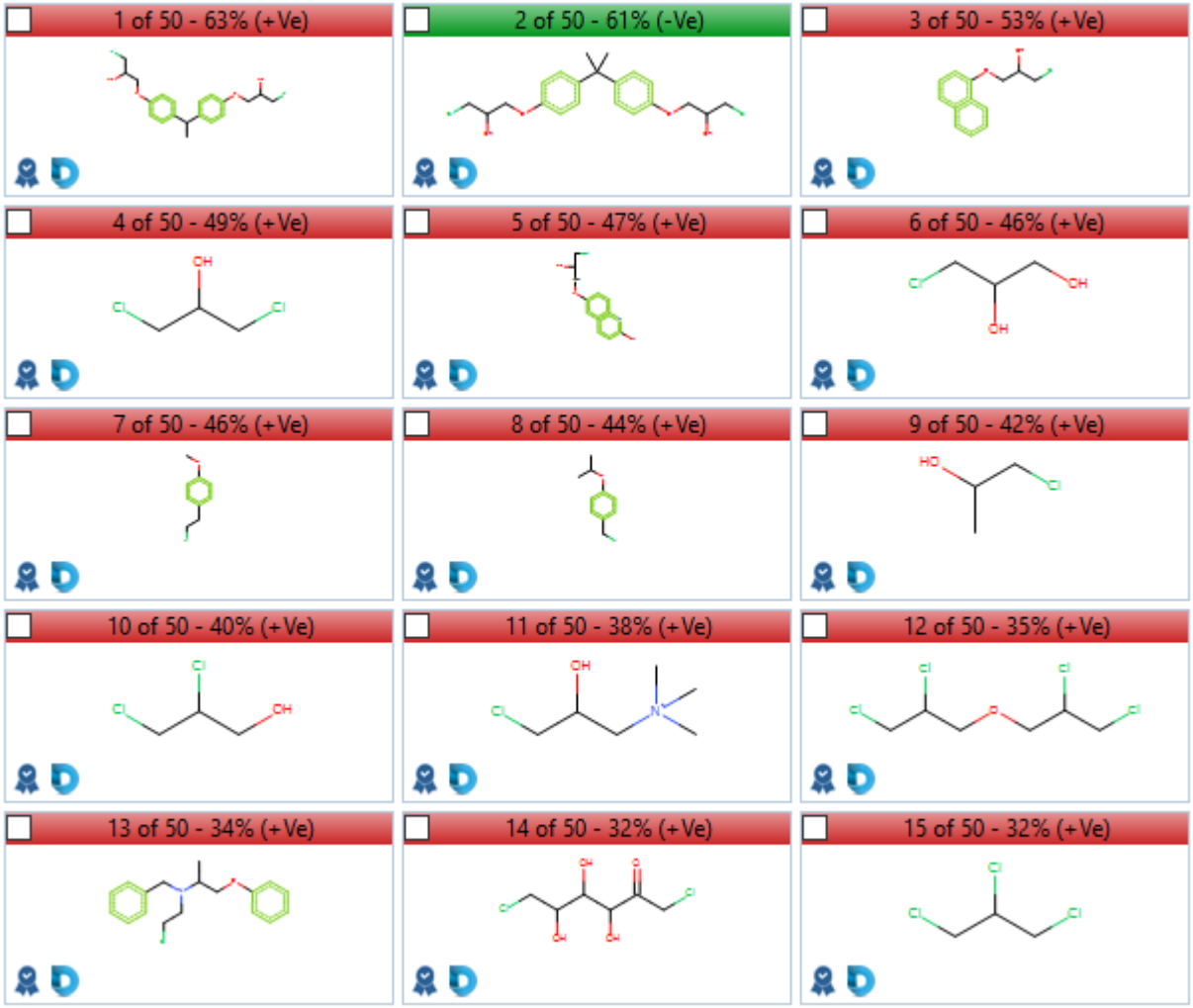
Figure 12. Result presented by the *in silico* mutagenicity prediction for atenolol impurity D.



Although the confidence of Sarah's prediction was low (3%), the alkyl halide alert could not be refuted through expert review. From the first 15 nearest neighbors shown through Sarah's interface which contained the same alkyl chloride moiety (Figure 13), only one compound was negative (61% similarity with Chlorhydrin). Hence, this impurity was assigned to class 3.

However, considering that this impurity is mentioned in a pharmacopoeial monograph with a limit higher than the TTC, the Ames test was conducted to confirm the bacterial mutagenicity of this compound, and the result was positive, as described in section 5.3.1. Hence, the *in silico* systems were capable of accurately predicting that this would be a mutagenic compound.

Figure 13. Nearest neighbours from Sarah interface which contain the alkyl chloride moiety



5.6 PRACTICAL EXAMPLES OF THE RISK ASSESSMENT

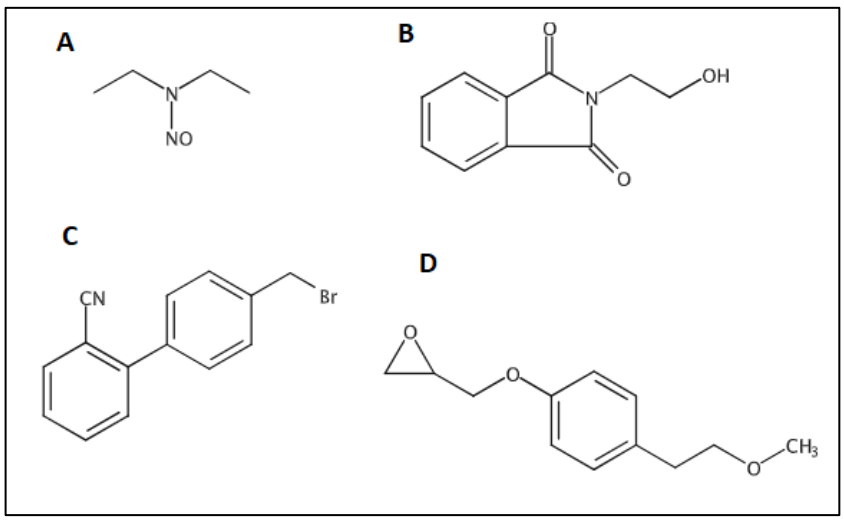
Five examples of the risk assessment are presented in this section, aiming to cover the different classes and different possibilities of approaches for the exposure evaluation and control strategies. For each impurity identified the classification was performed using the *in silico* systems as recommended in ICH M7. For classes 1, 2 and 3 the risk assessment was further conducted, and the control strategies were defined. A summary of the classification of these impurities is presented in Table 15, and the chemical structure of the impurities are represented in Figure 14.

Table 15. Summary of classification of impurities presented in cases 1-5.

Case	Impurity	CAS number	Derek Prediction	Sarah Prediction	Similarity to API*	Overall Carcinogenicity	Overall Ames	ICH M7 Class
1 and 2	N-nitroso diethylamine (NDEA)	55-18-5	Plausible: Alert007 – N-Nitro or N-nitroso compound	Positive – 100%	Alert(s) not found in API	Active	Active	1
3	N-(2-hydroxyethyl) phthalimide	3891-07-4	Inactive: No misclassified or unclassified features	Negative – 33%	No Derek Alerts found	Unspecified	Unspecified	5
4	Bromomethyl cyano biphenyl	114772-54-2	Plausible: Alert027 – Alkylating agent	Negative – 24%	Alert(s) not found in API	Unspecified	Unspecified	3
5	2-[[4-(2-methoxyethyl) phenoxy]methyl]-oxirane	56718-70-8	Plausible: Alert349 – Glycidyl ether, amine, ester or amide	Positive – 47%	Alert(s) not found in API	Unspecified	Unspecified	3

*API = Active pharmaceutical ingredient (drug substance)

Figure 14. Structures of impurities described in cases 1-5. (**A**): N-nitrosodiethylamine (NDEA), (**B**): N-(2-hydroxyethyl) phthalimide, (**C**): Bromomethyl cyano biphenyl, (**D**): 2-[[4-(2-methoxyethyl) phenoxy]methyl]-oxirane

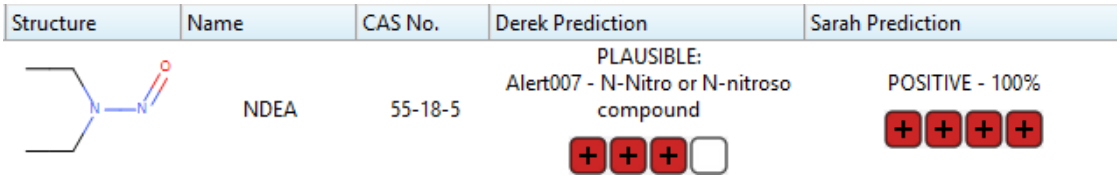


5.6.1 CASE 1

The impurity is N-nitrosodiethylamine (NDEA). This is a known mutagenic carcinogen, hence classified as class 1, besides being in the cohort of concern. The *in*

silico prediction gave the following result: a plausible prediction by Derek Nexus, which fired the N-nitroso alert; and a positive outcome for Sarah Nexus, with 100% confidence (Figure 15). This means that the searched impurity is within the software database, hence an Ames test has already been conducted for this compound, yielding a positive result. A positive result has also been obtained for this impurity in a carcinogenicity study, which is included in Lhasa's Carcinogenicity Database (https://carcdb.lhasalimited.org/carcdb-frontend/). This database gathers many carcinogenicity studies and provides the corresponding TD_{50} for each one.

Figure 15. Result presented by the *in silico* mutagenicity prediction for NDEA.



For calculation of the acceptable limit, the most relevant TD_{50} must be used. Since in this case several carcinogenicity studies are available, in various species, the species for which a greater number of studies was done and with the highest number of tumor sites, which is the rat, was chosen for extrapolation of the limit. The TD_{50} as per Gold standard calculation is 0.026 mg/kg/day (LHASA LIMITED, 2019). This means that a dose of 0.026 mg/kg/day would cause a cancer incidence of 1:2 in rats. To obtain the acceptable limit in humans, this value must be divided by 50,000 to obtain a negligible risk (1:100,000) and multiplied by 50 kg to account for a conservative estimate of the body weight of a human adult (ICH M7 (R1), 2017). Hence in this case, the acceptable intake of the impurity is 0.026 µg/day. Since the maximum daily dose of this drug substance is 150 mg/day, the maximum acceptable limit for the impurity in the drug substance is 0.17 ppm.

As described in section 5.5, this impurity is generated in the route of synthesis of the starting material, that is, early in the process. Close to the stage where the tetrazole ring is formed, sodium nitrite is used along with hydrochloric acid, hence providing nitrous acid. In the sequence, triethylamine is used, which may be contaminated with diethylamine, hence allowing for the possibility of N-nitrosodiethylamine (NDEA) to be formed upon contact with nitrous acid (Figure 16). The initial concentration of NDEA was estimated based on theoretical values,

considering the quantities of sodium nitrite input and the purity of the triethylamine used. The drug substance manufacturer ensured that there was no possibility of generation of NDEA from any other sources, showing compliance with good manufacturing practices (GMP) in their operations, hence avoiding cross-contamination from other processes or the origin of the precursors in other materials used in the process. Considering this scenario, the purge factor calculation was performed first for nitrous acid (Table 16) to estimate how much would be available to react with diethylamine, and then for NDEA considering the initial concentration of its precursors (Table 17).

Table 16. Purge factor calculation for nitrous acid.

Stages of the manufacturing process		Reactivity	Solubility	Volatility	Technical rationale	Purge factor
1	Water washing after formation of nitrous acid	1	10	1	Nitrous acid is soluble in water while the intermediate is soluble in organic phase	10
2	Washing with toluene	1	1	1	Nitrous acid is not soluble in toluene hence not purged	1
3	Drying	1	1	1	Nitrous acid's boiling point is 158ºC, which is higher than the drying temperature hence it does not volatilize	1
4	Reaction – tetrazole formation	1	1	1	In this stage, triethylamine is added, yielding the formation of NDEA	1
					Nitrous acid predicted purge factor	**10**

Table 17. Purge factor calculation for NDEA.

Stages of the manufacturing process		Reactivity	Solubility	Volatility	Technical rationale	Purge factor
5	Acid extraction	1	10	1	NDEA may protonate and stay in the aqueous phase while the intermediate remains in the organic phase	10
6	Extraction with water/organic solvent	1	10	1	NDEA is still protonated and once again separated from the intermediate	10
7	Distillation	1	1	1	The boiling point of NDEA is 177ºC, which is higher than the temperature of this distillation, hence it does not volatilize	1
8	Washing with methanol and centrifugation	1	1	1	Both NDEA and the intermediate stay in the organic phase and are not separated	1
9	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
10	Reaction to obtain next intermediate	1	1	1	NDEA does not react	1

11	Distillation	1	1	1	The temperature is much lower than 177ºC, hence NDEA does not volatilize	1
12	Precipitation of the intermediate	1	10	1	The intermediate precipitates while NDEA remains in the mother liquor, hence they are separated	10
13	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
14	Washing with organic solvent	1	10	1	The solid intermediate is washed, and NDEA is soluble in the washing solvents, hence removed	10
15	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
16	Reaction to obtain next intermediate	1	1	1	NDEA does not react	1
17	Extraction with water/organic solvent (3x)	1	10	1	The intermediate remains in the organic layer, while NDEA is soluble in water, hence removed in this step.	10
18	Reaction to obtain next intermediate	1	1	1	NDEA does not react	1
19	Extraction with water/organic solvent (3x)	1	10	1	The intermediate remains in the organic layer, while NDEA is soluble in water, hence removed in this step.	10
20	Precipitation of the intermediate	1	10	1	The intermediate precipitates while NDEA remains in the mother liquor, hence they are separated	10
21	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
22	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
23	Slurry and precipitation of the intermediate	1	10	1	The intermediate is dissolved and again precipitated while NDEA remains in the mother liquor, hence they are separated	10
24	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
25	Washing with organic solvent	1	10	1	The solid intermediate is washed, and NDEA is soluble in the washing solvents, hence removed	10
26	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
27	Slurry and precipitation of the intermediate	1	10	1	The intermediate is dissolved and again precipitated while NDEA remains in the mother liquor, hence they are separated	10

28	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
29	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
30	Slurry and precipitation of the intermediate	1	10	1	The intermediate is dissolved and again precipitated while NDEA remains in the mother liquor, hence they are separated	10
31	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
32	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
33	Reaction to obtain next intermediate	1	1	1	NDEA does not react	1
34	Filtration and washing with water	1	1	1	NDEA remains in the aqueous phase, as well as the intermediate, and is not purged	1
35	Extraction with water/organic solvent	1	1	1	NDEA remains in the aqueous phase, as well as the intermediate, and is not purged	1
36	Precipitation of the intermediate	1	10	1	The intermediate precipitates while NDEA remains in the mother liquor, hence they are separated	10
37	Centrifugation	1	1	1	This operation physically separates the solid intermediate from the soluble NDEA as a consequence of the previous step	1
38	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
39	Washing with water	1	10	1	The solid intermediate is washed with water, hence NDEA is removed	10
40	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
41	Centrifugation	1	10	1	More water is added, and the centrifugation step is repeated. Additional NDEA if present will be removed since it is soluble in water	1
42	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
43	Washing with water	1	10	1	The solid intermediate is washed with water, hence NDEA is removed	10
44	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1

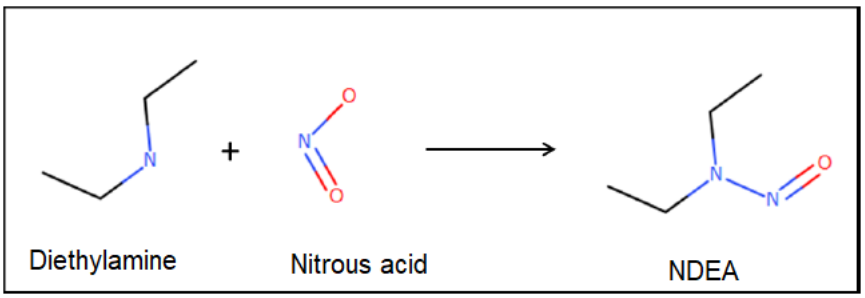
45	Centrifugation	1	10	1	More water is added, and the centrifugation step is repeated. Additional NDEA if present will be removed since it is soluble in water	1
46	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
47	Washing with water	1	10	1	The solid intermediate is washed with water, hence NDEA is removed	10
48	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
49	Reaction to obtain the drug substance	1	1	1	NDEA does not react	1
50	Carbon treatment and filtration	1	1	1	This step removes solid particles, NDEA is not purged	1
51	Distillation	1	1	1	The temperature is much lower than 177ºC, hence NDEA does not volatilize	1
52	Crystallization of the drug substance	1	10	1	The solid drug substance is separated from NDEA which is soluble in the mother liquors	10
53	Centrifugation	1	1	1	This operation physically separates the solid drug substance from the soluble NDEA as a consequence of the previous step	1
54	Washing with organic solvent	1	10	1	The solid drug substance is washed, and NDEA is soluble in the washing solvents, hence removed	10
55	Drying	1	1	1	The drying temperature is much lower than 177ºC, hence NDEA does not volatilize	1
					NDEA predicted purge factor	**10^{17}**
					API dose (mg)	150
					Acceptable limit (ppm)	0.17
					Required purge factor	25717
					Purge ratio	3.88×10^{12}

In this case, the predicted purge factor was 10 for nitrous acid and 10^{17} for NDEA, an extremely high value. The required purge factor was calculated as 25,717, which is derived from NDEA initial concentration divided by the acceptable limit. The initial concentration is derived from the concentrations of its precursors. The input of nitrous acid in the process was 20102 ppm. In the stage where it could react with diethylamine, its concentration had already been reduced by a factor of 10, yielding a theoretical concentration of 2010 ppm in the medium. As a conservative approach, the initial concentration of diethylamine was estimated as 5000 ppm, since the specification of triethylamine ensures the assay is not less than 99.5%. If 0.5% (5000

ppm) of diethylamine was present in triethylamine – which is a worst-case scenario – this would be the initial concentration of diethylamine.

Nitrous acid and diethylamine react in a 1:1 molar ratio to yield NDEA, as shown in Figure 16.

Figure 16. Nitrosation of diethylamine to yield NDEA.



To calculate how much NDEA would be formed, the molar quantities must be considered, as described in Table 18.

Table 18. Quantities of reagents and product in the nitrosation reaction.

	Nitrous acid	Diethylamine	NDEA
Molar ratio for reaction	1	1	1
Molecular mass	47 g/mol	73.14 g/mol	102.14 g/mol
Concentration of reactants (ppm)	2010 ppm	5000 ppm	-
Concentration of reactants (µmol/L)	42.8 µmol/L	68.4 µmol/L	-

The reactant limiting the reaction is the one with the lowest concentration given in µmol/L, which in this case is nitrous acid. It has been reported that diethylamine could also be formed by degradation of triethylamine, which could theoretically lead to an initial concentration of diethylamine higher than 5000 ppm (EMA, 2019d). However, since nitrous acid is the limiting reactant, this would make no difference in the amount of NDEA to be generated.

Hence if the reaction occurs with 100% yield, 42.8 µmol/L of NDEA would be generated. Considering its molecular mass (102.14 g/mol), this represents 4,372 ppm of NDEA in this stage.

The required purge factor is the initial concentration (4,372 ppm) divided by the safe limit (0.17 ppm), hence 25,717. Finally, the purge ratio is calculated as 3.88×10^{12} (predicted purge factor divided by required purge factor), which is a value higher than 1,000. This supports the use of option 4 as the control strategy of NDEA in this case, since the process can purge the impurity at least 3.88×10^{12} times more than it would be needed to achieve the safe limit.

5.6.2 CASE 2

A second manufacturer for the same drug substance described in case 1 uses a different route of synthesis, where the formation of the tetrazole occurs in the last synthetic step of the process. In this case, sodium nitrite, hydrochloric acid and triethylamine are also the precursors of NDEA, however there are not enough steps to ensure its adequate and robust elimination in all batches. However, analytical data has been provided for 96 batches, showing compliance with the limit of 0.17 ppm. The risk assessment is hence concluded showing the risk is negligible.

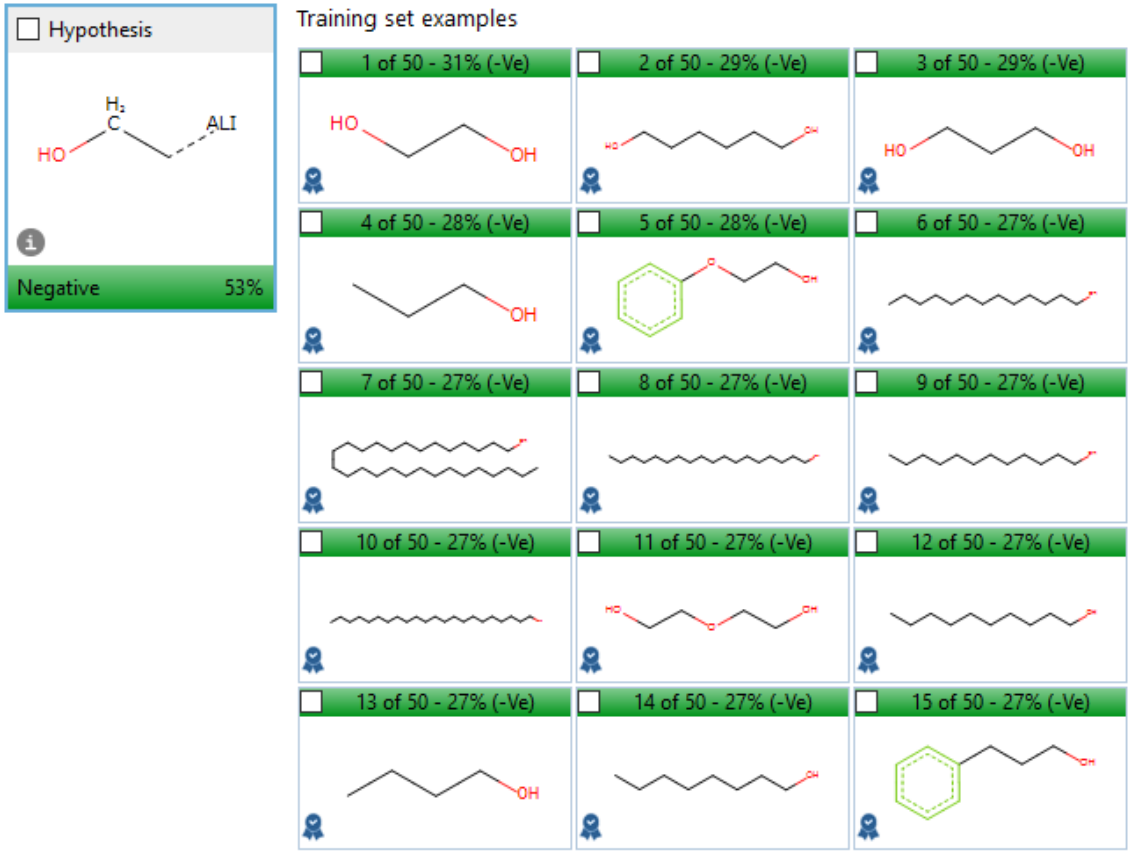
In this case, the purge factor could not be calculated due to the proximity of the impurity generation to the final drug substance in the process. In addition, analytical results of some batches were above 30% of the acceptable limit, so the control strategy chosen was option 1 – controlling the impurity in the drug substance release specification.

5.6.3 CASE 3

This impurity is named N-(2-hydroxyethyl) phthalimide and consists of an intermediate formed in early stages of the manufacturing process. The prediction was negative both by Derek and Sarah Nexus, indicating a high probability of this impurity being assigned as class 5. Nonetheless, an expert review was done to verify the accuracy of the prediction.

Derek did not fire any alert for this structure, hence no additional details were provided by this system. Sarah, on the other hand, presented two different hypotheses for this structure, along with the relevant examples related to each hypothesis (Figures 17 and 18).

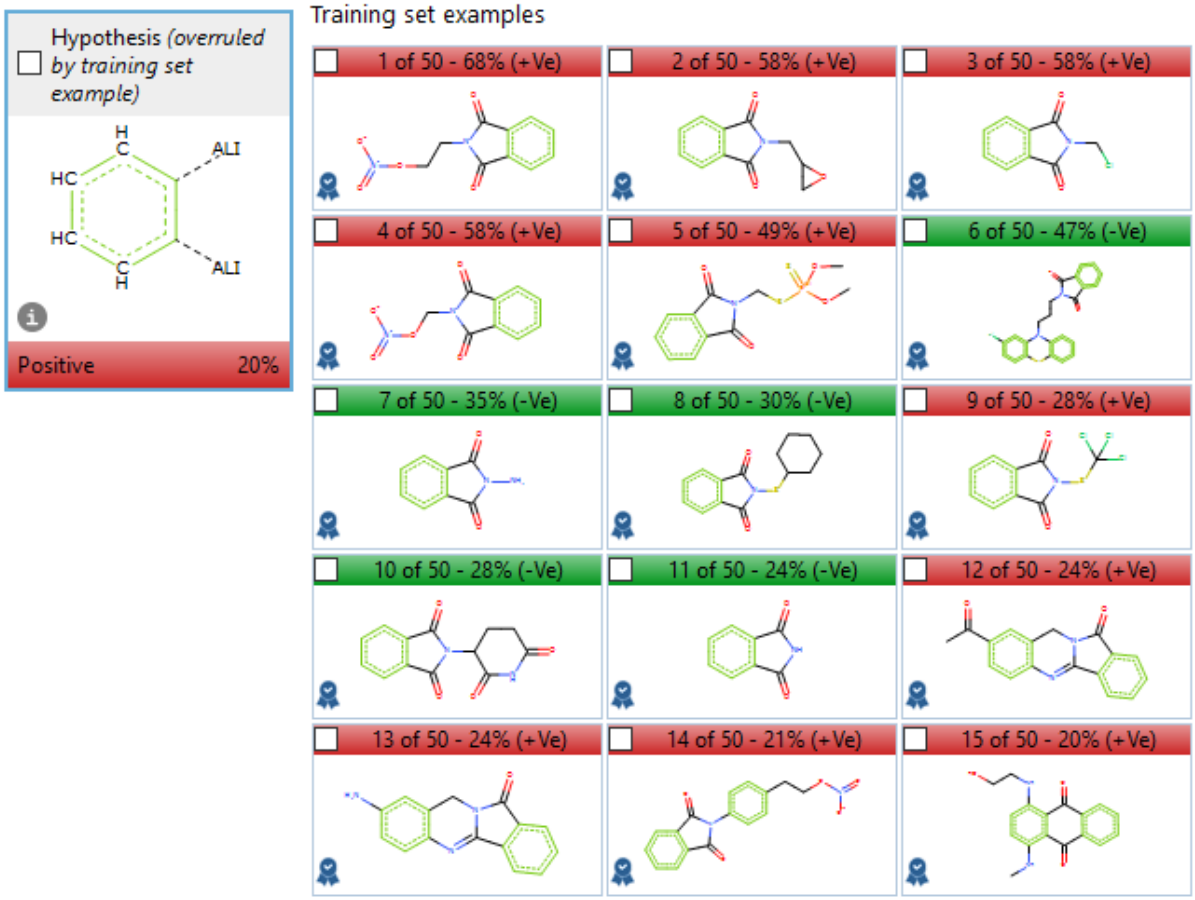
Figure 17. First hypothesis generated by Sarah Nexus for N-(2-hydroxyethyl) phthalimide, with 53% confidence, and related examples.



The overall confidence given by Sarah is the average of the confidence given for each hypothesis. In this case, the first hypothesis is negative with 53% confidence and the second one is positive with 20% confidence. The overall prediction is negative with 33% confidence.

The examples presented by Sarah related to each hypothesis must be evaluated in the expert review. These are compounds which are within the software database, that is, they have been tested in a bacterial mutagenicity assay, and their result is indicated by the color (green - negative or red - positive) assigned to them. The examples must be compared to the query structure to understand if they are relevant or not for the case.

Figure 18. Second hypothesis generated by Sarah Nexus for N-(2-hydroxyethyl) phthalimide, with 20% confidence, and related examples.



The first hypothesis presents a series of long chain compounds which show relatively low similarity with the impurity (most similar example shows 31% similarity). The examples shown in the second hypothesis are more similar to the query impurity, with the closest neighbor showing 68% similarity. While the second hypothesis is a positive outcome, this may raise concern on the relevance of Sarah overall conclusion. However, upon evaluation of the examples of the second hypothesis, it was concluded that the most similar positive compounds (Figure 18, compounds 1-5) show other mutagenicity alerts in their structure which are not relevant for the query impurity. Example 1 includes a nitro group in its structure, example 2 includes an epoxide, example 3 includes an alkyl halide, and so forth. Hence, these are not relevant examples to raise concern on the mutagenicity of the query impurity. When other examples are examined, which do not contain additional alerts when compared to the query impurity, their result is negative.

Hence, this is a class 5 impurity which is predicted to be not mutagenic. The risk assessment is finalized at this stage since ICH M7 controls do not apply for this impurity, and it can hence be handled as a regular organic impurity to be controlled under the scope of ICH Q3A/Q3B.

5.6.4 CASE 4

The impurity bromomethyl cyano biphenyl is a starting material used in the first stage of the manufacturing process. The *in silico* prediction gave conflicting results, since Derek predicted the compound to be positive, and Sarah predicted it to be negative. Hence in this case, the expert review is especially important to adequately assign a class for this impurity.

When Derek Nexus fires an alert for a structure, additional comments are provided regarding the mechanisms involved and the studies which generated that alert. In this case, the alkylating agent alert was fired, due to the alkyl bromide moiety of the structure. Sarah, on the other hand, identified the same part of the structure as its hypothesis, however, it gave a negative prediction based on the most similar examples of the database (Figure 19). At first, when Sarah's examples were evaluated, they seem to overrule Derek's alert, because many compounds containing the alkyl bromide moiety were negative in the mutagenicity assay. Yet, the below information was provided under Derek`s comments session:

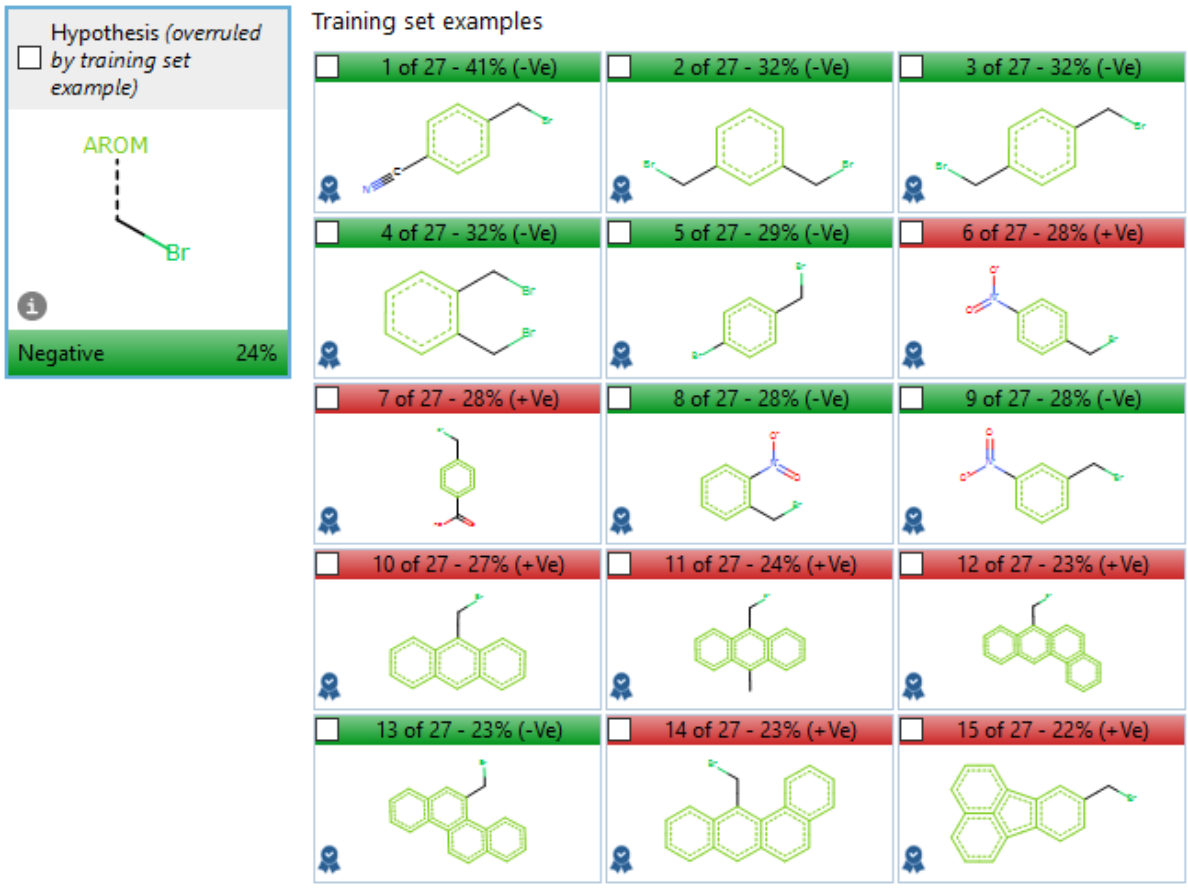
"There is also some evidence to suggest that the mutagenicity of some benzyl halides may not be observed in the Ames test. This may be due, in part, to their high cytotoxicity and inability to be tested at high doses."

"In contrast, corresponding biphenyl compounds, such 4-(chloromethyl)biphenyl and polyaromatic compounds such as 9-chloromethylanthracene and 1-chloromethylpyrene do give strong positive responses in the Ames test despite their toxicity."

Hence, these comments explain the reason why many examples presented by Sarah, which contain the alkyl bromide, are negative. They are benzyl halides which show cytotoxicity and cannot be tested at high doses, so these results may be biased. The query compound, on the other hand, is a biphenyl compound hence would be

expected to give strong positive responses in the Ames test. Therefore, this impurity has been assigned as class 3.

Figure 19. Hypothesis generated by Sarah Nexus for Bromomethyl cyano biphenyl, with 24% confidence, and related examples.



Once classifying the impurity as potentially mutagenic, the acceptable limit must be calculated. Since there is no carcinogenicity data for this impurity, a compound-specific limit cannot be generated, and the general TTC approach is used. Since the drug substance is an anti-hypertensive drug used for more than 10 years, the acceptable intake is 1.5 µg/day. The maximum daily dose of this drug is 320 mg/day, hence the limit is 4.69 ppm (acceptable intake in µg divided by maximum daily dose in g).

Since this impurity is generated early in the process, the purge factor calculation was performed, to assess whether the elimination of the impurity can be justified. The calculation and technical rationale are presented in Table 19.

Table 19. Purge factor calculation for bromomethyl cyano biphenyl.

Stages of the manufacturing process		Reactivity	Solubility	Volatility	Technical rationale	Purge factor
1	Reaction to obtain the first intermediate	100	1	1	The conditions are ideal for conversion of the starting material into the intermediate, hence the impurity is highly reactive.	100
2	Ionisation of the intermediate	1	10	1	The intermediate is ionised and precipitated while the impurity is not, hence it remains soluble. This separates one from the other.	10
3	Centrifugation	1	1	1	This step physically separates the impurity from the intermediate after the previous precipitation.	1
4	Drying	1	1	1	The temperature is not high enough to volatilize the impurity	1
5	Reaction to obtain second intermediate	1	1	1	The impurity does not react in this condition.	1
6	Solvent extraction	1	1	1	Both the impurity and intermediate are soluble in the medium.	1
7	Reaction to obtain third intermediate	1	1	1	The impurity does not react in this condition.	1
8	Solvent extraction	1	1	1	Product is extracted in organic layer and impurity is soluble in this same layer	1
9	Reaction to obtain the drug substance	1	1	1	The impurity does not react in this condition.	1
10	Solvent extraction	10	1	1	Upon addition of NaOH the impurity might react providing the benzylic alcohol derivative	10
11	Liquid-liquid extraction (alkaline)	1	10	1	The drug substance is soluble in aqueous layer, while the impurity will be soluble in the organic layer.	10
12	Liquid-liquid extraction (acidic)	1	1	1	The drug substance is soluble in organic layer due acidic pH, as well as the impurity.	1
13	Liquid-liquid extraction (Neutral)	1	10	1	The drug substance is soluble in aqueous layer, while the impurity will be soluble in organic layer	10
14	Liquid-liquid extraction (Acid)	1	1	1	The drug substance is soluble in organic layer due acidic pH, as well as the impurity.	1
15	Solvent distillation & isolation	1	10	1	The drug substance is not soluble in the solvent used, hence it is in solid form, separated from the impurity	10
16	Filtration	1	10	1	The same solvent is charged again, which will purge organic impurities	10

17	Liquid-liquid extraction (Base)	1	10	1	In basic extraction, the drug substance is soluble in aqueous phase and separated from organic impurities.	10
18	Liquid-liquid extraction (Base)	1	3	1	Same as previous extraction	3
19	Precipitation	1	10	1	The used solvent does not solubilize the drug substance, which precipitates from the reaction mass and so is separated from the soluble impurity	10
20	Filtration	1	1	1	Filtration physically separates the impurity from the intermediate after the previous precipitation	1
21	Drying	1	1	1	The temperature is not high enough to volatilize the impurity	1
					Predicted purge factor	**3×10^{10}**
					API dose (mg)	320
					Acceptable limit (ppm)	4.69
					Required purge factor	213219
					Purge ratio	140700

As described in Table 19, the predicted purge factor was 3×10^{10}. In order to obtain the purge ratio, this value must be compared with the required purge factor. Since in this case the impurity is the starting material, the initial concentration is considered to be 100% or 1,000,000 ppm. The required purge is thus 213,219 (initial concentration divided by safe limit which is 4.69 ppm). Hence, the purge ratio is 140,700. Since this value is much higher than 1,000, the elimination of the impurity is justified, and the risk assessment is concluded showing this impurity does not represent a risk. The control strategy adopted was option 4.

5.6.5 CASE 5

This impurity is 2-[[4-(2-methoxyethyl) phenoxy]methyl]-oxirane and it is the last intermediate before obtaining the drug substance in its route of synthesis. Both Derek and Sarah agreed to classify the impurity as potentially mutagenic. Derek alert was the glycidyl derivative, which contains an epoxide ring in the structure.

Sarah presented four different hypotheses and the overall call was positive with 47% confidence. As shown in Figure 20, the most relevant hypothesis considering the alert fired by Derek is the one involving the epoxide moiety. In order to refute the prediction given by Derek, a relevant example similar to the query and containing this

alert would need to be negative. Nonetheless, as shown in Figure 21, all examples which contained the epoxide in the structure were positive in the Ames test. Hence, the conclusion is that the impurity should be treated as mutagenic and is a class 3 impurity.

Figure 20. Four hypotheses generated by Sarah Nexus for 2-[[4-(2-methoxyethyl) phenoxy]methyl]-oxirane.

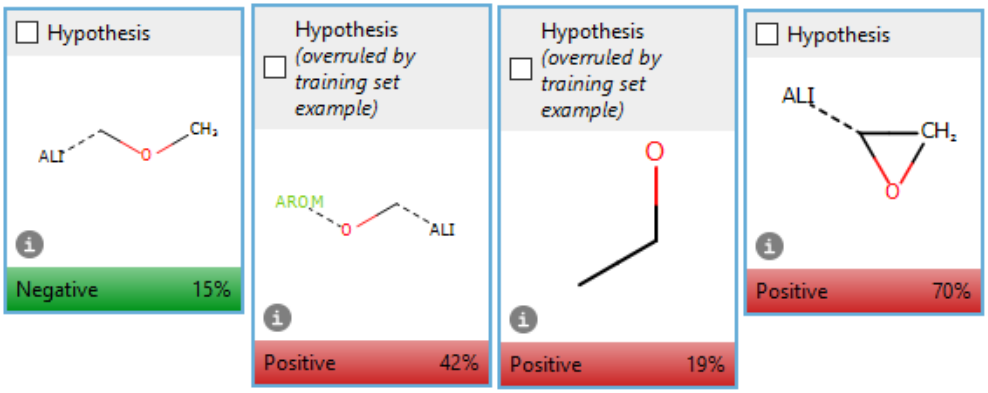
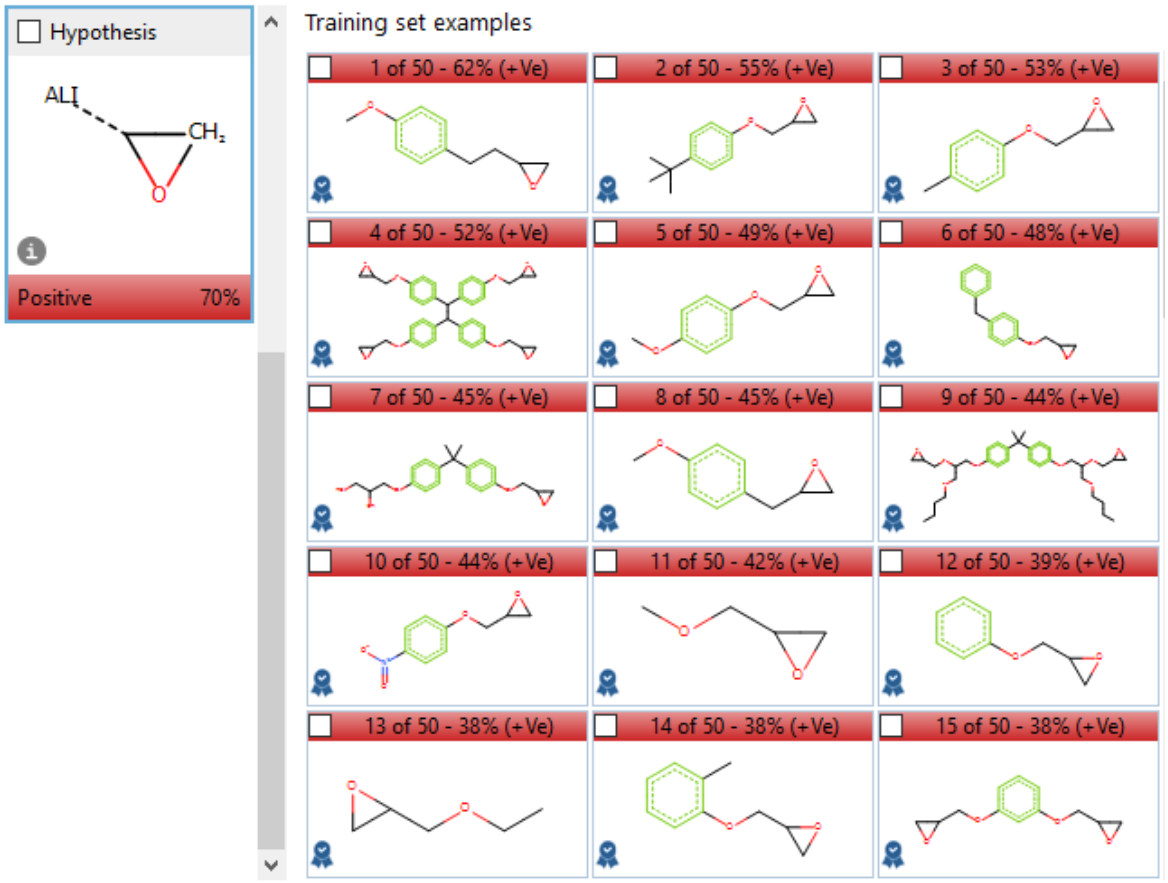


Figure 21. Epoxide hypothesis generated by Sarah Nexus for 2-[[4-(2-methoxyethyl) phenoxy]methyl]-oxirane and related examples.



The limit was calculated considering the TTC, 1.5 µg, and the maximum daily dose of this drug which is 400 mg. The acceptable limit is hence 3.75 ppm.

The drug substance manufacturer has presented trend analysis results with a validated analytical method, confirming the results of all batches comply with this limit. Hence, the risk assessment was concluded, and the risk is negligible.

Since the impurity is generated in the last synthetic step of the manufacturing process, and only a few purification stages are performed after it reacts to yield the drug substance, the purge factor is not a strong argument to ensure the impurity will be below the limit in all batches. Hence, a test was established in the drug substance specification, and the control strategy adopted was option 1.

6 DISCUSSION

6.1 PERCENTAGE OF POTENTIALLY MUTAGENIC IMPURITIES IN THE EVALUATED ROUTES

The 15 evaluated drug substances are anti-hypertensive drugs, which are among the most widely used drugs in Brazil (CMED; ANVISA, 2017). A recent publication showed that only 10 drug substances represent more than 90% of the anti-hypertensive treatments in Brazil (MENGUE et al., 2016). Six of these drugs have been included in this present evaluation, which proportionally represent more than 63% of the anti-hypertensive treatments in Brazil, based on this previous publication.

Among the 262 evaluated impurities, 57 showed mutagenic potential, which represent 22% of the impurities described by the drug substance manufacturers. This result agrees with a previous report by Delaney and coworkers which estimated that around 20-25% of the intermediates used in synthetic routes of drug substances would be positive in an Ames test (DELANEY, 2007).

At first, in this project it would be expected to find a lower amount of mutagenic impurities than described by Delaney and coworkers, considering that not only the intermediates and reagents from the route of synthesis were evaluated, but also other impurities described in the DMF, which may be reaction by-products or even degradation products. These compounds are expected to have a lower reactivity when compared to the drug substance, considering that the drug or synthetic intermediates usually react forming by-products and degradation products, and not the opposite. Because of that, it is not expected that these impurities would react with the DNA and exhibit mutagenicity in the same frequency as the reactive intermediates used in the route of synthesis. Hence, including these impurities in the study would theoretically lead to a lower percentage of impurities positive in the Ames test.

However, in this project the evaluation was performed through *in silico* tools, which are in general more conservative than the Ames test itself. For regulatory purposes, the expert-rule and statistical based systems are calibrated to yield higher false-positives, in order to reduce the false-negatives (DOBO et al., 2012). Impurities assigned to class 3, which were not tested in the Ames test but had a positive prediction for mutagenicity, could have their mutagenicity refuted in case they were

actually tested *in vitro*. Thus, it is expected that a greater number of potentially mutagenic impurities would be identified. Consequently, both factors compensate for each other and the percentage found was similar to the one previously described.

Another recent publication estimated that drug substance manufacturing processes with 6 synthetic steps would include around 4 DNA-reactive intermediates for each process (ELDER; TEASDALE, 2015). In this work, considering the 57 potentially mutagenic impurities identified in 22 manufacturing processes, an average of almost 3 DNA-reactive impurities were found for each process. This smaller number may be explained because the synthetic processes evaluated present in average 3 synthetic steps. Longer manufacturing processes would probably result in more potentially mutagenic impurities, however it is not common for 6 synthetic steps to be part of the route of synthesis presented in the open part of the DMF which is shared with customers. The paper by Elder and Teasdale is a revision of 300 publications, and it would be expected that scientific publications include more steps than the DMF elaborated for regulatory purposes by the drug substance manufacturer.

If an average of 6 stages were included in this present evaluation, it would be expected to find even more than 4 potentially mutagenic impurities per route. The previous publication identified reactive intermediates based on structural alerts only (ELDER; TEASDALE, 2015), so if two *in silico* tools were applied as required in ICH M7, this would probably result in an even higher number of potentially mutagenic impurities.

A greater number of mutagenic impurities is expected when previous stages are included in the evaluation because initial steps of the manufacturing process usually use more simple and reactive reagents, with higher probability of being mutagenic. On the other hand, the possibility of purging these reagents is extremely high, due to the many synthetic steps, work-up of reactions and purification of intermediates and drug substance done after their introduction.

Based on this rationale, ICH M7 requires that the mutagenicity of impurities be evaluated only after the introduction of the registered starting material of the process. Stages before the introduction of the starting material do not need to be evaluated, because it is considered that if the starting material was well selected, the route of

synthesis from its introduction will include enough purge steps, with many opportunities for elimination of impurities. Hence, impurities arising from previous stages are not considered relevant.

The starting material is the compound which defines the beginning of the route of synthesis from which good manufacturing practices (GMP) must be applied, as described in ICH Q7 (ICH guideline for good manufacturing practice for active pharmaceutical ingredients), because the steps after introduction of the starting material are considered to impact the drug substance quality and impurity profile. The definition of the starting material is part of a regulatory framework and should be based on the principles defined in ICH Q11 (ICH guideline on development and manufacture of drug substances), which requires enough steps to be described in the route of synthesis from the starting material onwards, so that control strategies can be considered appropriate. Too short synthetic routes are generally not considered adequate, but a minimum number of synthetic steps which should be performed after introduction of the starting material is not mentioned by the guideline. Processes with 2 or 3 synthetic steps may be appropriate, if the other requirements defined in the guideline are fulfilled and the impurities control strategy is clear (ICH Q11, 2012).

For this work, the evaluation of the manufacturing processes took into consideration the principles defined in ICH Q11, so that when the synthetic route was too short, the previous stages were also included in the assessment. However, this was not the situation for most cases.

6.2 MOST COMMON MUTAGENICITY ALERTS AND CLASS-SPECIFIC LIMITS

When the mutagenicity prediction given by Derek is a positive outcome, it describes the structural alert responsible for that prediction. However, the overall prediction considers both systems (Derek and Sarah), so in some cases the mutagenicity concern may be due only to data presented by Sarah, and Derek may not describe a specific alert. The most common alerts presented by Derek have been investigated for all potentially mutagenic impurities (classes 1, 2 and 3), and the results show that for impurities arising from anti-hypertensive drug substances, the most common alerts were alkyl halides (20%), nitrosamines (17%) and epoxides (12%).

Nitrosamines would be expected to be common, since ‘sartans’ are anti-hypertensive drugs that were included in this project. These impurities are by-products, not intentionally added to the process. They are also part of the cohort of concern described in ICH M7, which means they are more potent that most carcinogens, hence required to be controlled at lower levels than the TTC. In fact, a class-specific TTC for nitrosamines has been described by EMA, which proposes 18 ng/day as the level that represents a 95% probability that the intake of any nitrosamine would result in a negligible risk (EMA, 2020a).

Alkyl halides and epoxides on the other hand, are usually intermediates or reagents used in the synthesis, hence intentionally added. These compounds are reactive in the synthesis and are also capable of alkylating the DNA, which is their mechanism of mutagenicity. They are not in the cohort of concern, so at first the TTC can be applied for these impurities, when assigned to classes 2 or 3. However, in the case of monofunctional alkyl chlorides and bromides, they have been shown to be less potent than most carcinogens – an opposite case to nitrosamines. Hence, as described by ICH M7 in Note 5, it is possible to justify a limit 10 times higher than the TTC for monofunctional alkyl chlorides (ICH M7 (R1), 2017). For monofunctional alkyl bromides, although not yet specified in the guideline, there is also evidence of their lower potency so the same approach may be used (BERCU et al., 2018).

One question that now arises is regarding the limit for epoxides. Since they are so common in these types of synthesis, representing 12% of the impurities with a mutagenic potential in this study, would it be possible to also establish a class-specific TTC for them? This is something that would be interesting to be investigated in the future, so that more realistic limits could be established instead of simply defaulting to the conservative TTC.

6.3 CONTROL STRATEGIES ADOPTED BY MANUFACTURERS

Regarding the control strategies, the initial observation was that many drug substance manufacturers had not yet chosen between one of the four options described in ICH M7, based on the drug master file. In many cases, only after communication with the manufacturer was a control strategy established.

Since the application of ICH M7 is only intended for new drug products to be registered or when there are significant changes in the manufacturing processes, it is not a regulatory requirement for approved drug substances, unless a known carcinogen or mutagen is identified. The fact that many manufacturers did not have a control strategy in place before they were requested to confirms the hypothesis that it would be appropriate for ICH M7 to be applicable to marketed drugs, which would ultimately ensure that adequate control strategies would be proactively in place. Nonetheless, all manufacturers were aware of the guideline and committed to comply with the requirements once requested. Thus, as a result of this project, ultimately a control strategy was chosen for each potentially mutagenic impurity identified.

The most common control strategy was option 4 (no analytical control, 60%), followed by option 1 (analytical control in the drug substance, 28%). Interestingly, fewer cases (7 impurities, 12%) consisted of controlling the impurity in an intermediate of the process. The controls applied to intermediates are confidential information of the manufacturer which are usually not disclosed in the open part of DMF which is shared with customers. However, to ensure compliance with ICH M7, such information must be presented somehow.

For these 7 impurities, option 3 was used, so the limit for the impurity in the intermediate was higher than the acceptable limit in the drug substance. No manufacturer chose to use option 2, where the same limit which would be acceptable in the drug substance is applied to the impurity in the intermediate stage.

One hypothesis to explain this is that the control of mutagenic impurities usually requires sensitive and more complex analytical methods, especially when using the original limit (option 2), while these methods may not be considered suitable for in-process controls. In some cases, the drug substance manufacturer does not own a mass spectrometer and this kind of analysis must be done at a third-party laboratory. In such cases, the results may take a longer time to be released, which may not be desired when a manufacturing process needs to wait for that result to move forward. This may be one of the reasons why manufacturers prefer to control these impurities in the drug substance specification, rather than in intermediate specifications.

Nonetheless, the first response of most manufacturers was not to control the impurities in any specification, but to justify their elimination based on analytical results of industrial batches. As an attempt to adopt control option 4, they presented results showing that the level of the impurity was below 30% of the acceptable limit in usually only 3 batches. However, the purge factor calculation had not been applied. This behavior shows how the purge factor rationale is relatively new and not yet widely spread across all drug substance manufacturers.

According to Anvisa, 78% of the manufacturers for drug substances used in drug products approved in Brazil are situated in Asia (ANVISA, 2019b). In this work, 86% of the processes evaluated were performed by drug substance manufacturers from Asia. For this region, the purge factor is a new approach. Currently most publications on this subject are from the European Pharmaceutical Industry, and many manufacturers included in our study became familiarized with the rationale only after these publications were shared with them. This emphasizes the relevance of this project, which also has the aim to spread this knowledge to other regions, helping with the implementation of ICH M7 where most drug substance manufacturers are situated. For this purpose, part of this project was also to prepare scientific videos and share them with manufacturers. This has further helped their understanding of the purge factor, besides sharing the original scientific publications. The link to such videos is provided in the annex section.

Results in sequential batches show that the exposure is below the acceptable limit in those specific batches, but not that all batches will have the same result. If such results are presented, ICH M7 may allow for periodic testing to be performed instead of routine testing, but it does not exempt the manufacturers from including a test in a specification.

For option 4 to be considered appropriate, a detailed scientific rationale must confirm that the process is robust to ensure the elimination of the impurity in all batches. Therefore, the purge factor calculation is highly recommended.

Besides the low familiarity with the purge factor calculation, another possible explanation for manufactures choosing to adopt option 4 based on analytical results from industrial batches only is that their process was validated. The process validation

performed by drug substance manufacturers consists of the production of 3 industrial batches, where the impurity levels are tested ensuring that they are below the acceptable limits. In fact, this strategy may be considered appropriate for some residual solvents which are not used in the last steps of the process. If results in 3 batches confirm that the level of the solvent is below 10% of the limit, there is no need to perform a routine analytical test for this solvent (EMA, 2013). However, for mutagenic impurities a more conservative approach is necessary, considering their higher toxicity and lower limits, which may allow for greater variation of results to be obtained.

The flexibility given for residual solvents may also be explained based on their inherent volatility. Such compounds have more opportunities to be removed throughout the manufacturing process which may include different stages where temperatures higher than their boiling point are used. However, this strategy is not applicable for solvents used in the last stage of the process, which have less chances of being removed.

In the case of mutagenic impurities, they are not always volatile, so the use of this approach is questionable. Analytical results showing the levels of the impurity in sequential batches prove that the chances of finding the impurity are low, but do not ensure the reproduction of these results in the next batches to be produced. A recently published Questions and Answers document concerning ICH M7 also emphasizes that analytical results only are not sufficient to support the adoption of control option 4 (ICH M7 Q&A, 2020). Hence, the purge factor calculation is the most widely recommended approach to justify the elimination of such impurities, besides saving time and resources which would be invested in analytical testing by the manufacturer. Although not all mutagenic impurities are volatile, most of them are highly reactive, which is one of the greatest contributors to their elimination. In many cases, these are compounds used as reagents in initial stages of the route of synthesis, which allows them to be eliminated throughout the process, not only through reactivity, but also through solubility, and volatility when applicable.

Compounds arising from stages close to the final drug substance have less chances to be eliminated, especially if their structure is highly similar to the drug substance, which may also cause similarities in solubility and volatility between them.

In these cases, the reactivity may be the only factor which can reduce the levels of these compounds, and this may not always be enough considering the low levels acceptable for mutagenic impurities. Therefore, just like the rationale applicable for residual solvents, mutagenic impurities generated in the last stage of the process should generally be controlled through a test in the drug substance specification with appropriate limits, unless otherwise justified (ICH M7 (R1), 2017).

Although the first strategy chosen by manufacturers was to use option 4 based on analytical results of sequential batches, when they realized that more information would be needed to calculate the purge factor, most preferred to apply option 1. This would require less efforts in scientific terms, although it requires more time and resources for analytical testing.

The recommendation then given to manufacturers was to adopt option 1 for impurities generated in the last synthetic step, but to apply the purge factor calculation for impurities arising from early stages.

6.4 COMPARISON OF *IN SILICO* PREDICTIONS VS. EXPERIMENTAL RESULTS

In this project, a comparison of *in silico* predictions with experimental results was also performed. *In silico* tools have emerged as alternatives to reduce the use of animals in research and to increase the capacity to assess the safety of thousands of chemicals reaching the market, an initiative which has gained more attention in the last years (FORD, 2016). Few countries disclose statistics about the number of animals used in research (TAYLOR et al., 2008), however in the United States, the country where the most studies in animals are performed in the whole world (STATISTA, 2018), the statistics show that the number of animals used in research has been decreasing along the years (SPEAKING OF RESEARCH, 2017). Moreover, as a result of the efforts towards application of the 3Rs (replacement, refinement and reduction) relating to the use of animals in research (RUSSELL; BURCH, 1959), the US Environmental Protection Agency (EPA) has committed to eliminate all mammal testing by 2035 (US EPA, 2019).

Many other initiatives have emerged supporting the use of *in silico* models in different frameworks. Some examples are the European Union's REACH regulation, the Toxicology in the 21st Century (Tox21) program, and the International Cooperation on Alternative Test Methods (ICATM) (EU, 2006; EUROPEAN COMMISSION, 2020; MYATT et al., 2018; NTP, 2019).

The advantages of *in silico* methods go beyond avoidance of the use of animals in research. A study evaluated the benefits of using read-cross and *in silico* techniques using two voluntary high-production-volume (HPV) chemical programs to evaluate substances based on structural similarities, and found that upon evaluation of 261 chemicals, they avoided the use of 100,000 to 150,000 test animals, and saved US$50,000,000 to US$70,000,000 (STANTON; KRUSZEWSKI, 2016).

The low cost and reduced time to evaluate toxicity are some of the other advantages of *in silico* tools when compared to *in vivo* and *in vitro* studies (VALERIO, 2009). The Ames test, used to evaluate the bacterial mutagenicity of compounds, requires more than a month to be performed according to the OECD guideline and GLP principles, and presents a significant cost for each compound to be evaluated. Using *in silico* systems, the mutagenicity prediction can be obtained in a few seconds and with no additional cost to the user, once the license to the software has been purchased.

In this project, two stages of the risk assessment were performed with *in silico* tools: the identification of mutagenic impurities (hazard identification), and the evaluation of the level of the impurity present in the drug substance through the purge factor (exposure evaluation).

The validation of *in silico* methods for mutagenicity prediction have already been shown in various publications, which explains their acceptance by regulators and the fact that they are mentioned in ICH M7 (BARBER et al., 2016; DOBO et al., 2012; HONMA et al., 2019). These systems are highly conservative and usually overestimate the mutagenic potential of the tested impurities, leading to a higher number of false-positives to minimize the number of false-negatives. One study showed that the *in silico* systems were able to accurately predict a negative result in 94% of the cases. When the *in silico* analysis was combined with the expert review, the negative

predictivity increased to 99% (DOBO et al., 2012). Since the most critical when it comes to patient safety is the reliability of negative predictions (WILLIAMS et al., 2016), this degree of conservatism strengthens the trust that regulators have on these tools and reaffirms the applicability of computational toxicology in the prediction of mutagenicity of impurities.

However, in order to complement this study, an additional comparison of one *in silico* prediction was done with the experimental result given by the Ames test. In some instances, the predictions given by the softwares are inconclusive, and a classification cannot be established even after the expert review (AMBERG et al., 2019). In these cases, the conservative approach is to consider the impurity as class 3. However, this can be changed if a mutagenicity assay is conducted, which reclassifies the impurity in either class 2 (positive result) or 5 (negative result).

The case selected to have the mutagenicity prediction compared with the Ames test result was the compound 2-[4-[(2RS)-3-chloro-2-hydroxypropoxy] phenyl]-acetamide. This is impurity D described in the European Pharmacopoeia (EP) monograph of atenolol. The limit for this impurity as described by EP is 0.10%, and this was the limit originally adopted by the drug substance manufacturer as well. However, if this impurity was confirmed to be a mutagen, this limit would not be appropriate for this impurity.

The Ames test result confirmed this was a mutagenic impurity, and the mechanism for mutagenicity was shown to be the same as expected for this class of compounds. Based on the information provided by Derek Nexus, the trend observed for most alkyl halides is a mutagenic activity in the presence and absence of S9 mix, especially in *Salmonella typhimurium* TA100 and TA1535 (BARBER; DONISH; MUELLER, 1981; ERIKSSON et al., 1991). Impurity D was shown to be mutagenic in TA1535 and TA1537 with and without S9, but the mutagenic activity was not observed for TA100 – a 1.7-fold increase was observed only for the highest concentration. Although this is uncommon for alkylating agents, some other compounds have indeed been described to be positive only for TA1535 and not TA100 (WILLIAMS et al., 2019).

Hence, considering impurity D as a mutagenic impurity, its acceptable limit must take into consideration the appropriate TTC and the maximum daily dose of the drug

substance. Since this is an alkyl chloride, a limit 10 times higher than the TTC is justified (ICH M7 (R1), 2017). Therefore, the acceptable intake for impurity D is considered 15 µg/day, while the maximum daily dose of atenolol is 100 mg/day based on the drug product leaflet. This means the acceptable limit is 150 ppm or 0.015% (15 µg divided by 0.1 g). Nonetheless, the limit described in EP monograph for impurity D (0.10%) is higher than the ICH M7 calculated limit. This then suggests the need for the monograph to be revised.

This comparison showed that, as expected, the mutagenicity prediction was correct to consider this as a potentially mutagenic impurity, once again reiterating the accuracy of the *in silico* systems for mutagenicity prediction.

The use of the purge factor to predict the level of the impurity that would be expected in the drug substance, on the other hand, is not such a widespread concept when compared to the mutagenicity predictions. The comparison of the purge factor predicted levels with the experimentally determined levels has been shown by some publications but is still limited (BURNS et al., 2019, 2020; TEASDALE et al., 2010).

Hence, in this project, the prediction of the level of the impurity in the drug substance obtained using the purge factor was compared with the analytical results. Both results show that the impurity was appropriately eliminated, however the sensitivity limitation of the analytical method used to quantify the impurity reduced the effectiveness of this comparison. Since the purge factor prediction was that the impurity level was below 4×10^{-14} ppm, and the limit of detection of the analytical method used to test the impurity was 0.008 ppm, this comparison lead to the conclusion that the impurity level was in fact less than 0.008 ppm, but it was not possible to confirm how close it was to the predicted value.

A more efficient way to confirm the purge factor prediction would be using spike and purge experiments. Such studies are performed through intentionally adding known amounts of the impurity to a stage and measuring it some stages later. The difference between the initial and final concentrations provide the extent to which the stages in between actually purged the impurity. However, such a study requires the production of one batch that must be representative of the routine production of the

drug substance (BARBER et al., 2017). Since the addition of the impurity is not part of the validated process, this is not a regulatory batch which could have commercial use for the manufacturer. Hence, this type of study may not be considered advantageous by the manufacturers. Nonetheless, as described in question 8.6 of the ICH M7 Questions and Answers document, spiking experiments can be done in laboratory scale batches (ICH M7 Q&A, 2020). This flexibility then makes this a more feasible study to be conducted by manufacturers.

This present study showed that despite the inherent sensitivity limitation of the analytical methodology, the purge factor was able to predict that the analytical results would be the non-detection of the impurity.

6.5 CHALLENGES WHEN DEVELOPING ANALYTICAL METHODS

Developing analytical methods is a task that requires time and resources. Analytical standards must be acquired, and the process for their purchase usually takes a few months since most standards need to be imported. This period may be longer than the analytical validation itself. Moreover, any equipment needed for analytical methods is subject to instabilities and limitations.

In this project an analytical method was developed to determine the level of two potentially mutagenic impurities in nitrendipine, since the purge ratio obtained was not high enough to justify their elimination without additional experiments. Some of the challenges encountered, besides the long time for arrival of standards, were due to equipment limitations. During one of the analyses performed, there was overpressure in the UHPLC system, so that the analysis had to be repeated. Another limitation was that the Q-TOF-MS system was shut down for a period during this project, and when it was turned on again, the process for the vacuum to achieve the desired level took more than a week.

These obstacles increase the time needed for the analyses, which is already quite long. Nonetheless, the analytical burden in this project was less than would be needed for a quantitative method to be developed and validated. The intent of this method was to evaluate whether the levels of impurities were above or below a specific

limit, so instead of performing a complete validation which would be applicable to a quantitative method, a limit test validation was applied, including only specificity and limit of detection. This was possible because the level of the impurities were below 30% of the acceptable limit, which combined with the purge ratio allow for option 4 control strategy to be adopted - so a quantitative test did not need to be included in any specification. However, if the levels were found to be more than 30% of the acceptable limit, indicating a need to provide quantitative results, a complete validation should be done, including accuracy, linearity, precision, which would require additional experiments to be conducted.

Another challenge associated with including a test in the drug substance specification, especially when considering mutagenic impurities, is the need for sensitive equipment to be available at the quality control laboratory. Potentially mutagenic impurities have limits lower than the ones applied to regular impurities, meaning that a HPLC-UV system will usually not be appropriate to achieve the required limit of detection. In the case presented here, a UHPLC-Q-TOF-MS system was used, since the sensitivity level required for the impurities was in the ppm range. The aim was to achieve a detection limit at 30% of the acceptable limit, which represents 11.25 ppm of impurities in the sample. Since the sample concentration was 0.2 g/ml, the impurities solution prepared was a 2.25 ppm solution.

Mass spectrometers are sensitive equipments, however they are also more expensive and usually available only at the analytical development department in most companies. Today, they may not always be available at a quality control department for routine analysis of drugs. However, this seems to be an inevitable consequence for the near future, once the control of potentially mutagenic impurities is implemented, especially for cases when purging of the impurities cannot be justified.

The obstacles when dealing with analytical methods go even further than time, resources and cost. When considering mutagenic impurities, avoiding analytical methods should always be considered as the first strategy, to avoid exposure of the analyst to significant amounts of such toxic compounds. Besides, these compounds require special care for their disposal, so avoidance of their use is also a more sustainable approach, protecting the environment.

Hence, adopting control option 4 and applying the purge factor calculations should always be the first strategy when deciding how to control impurities. In this project the analytical method was developed with the aim to complement the purge rationale, since the calculation itself was not robust enough, but this should be avoided whenever possible. Purging calculations not only present many advantages over analytical methods, but were shown in this project to be reliable and to adequately substitute experimental results. This reinforces the importance of applying this calculation which is shown to be protective of the health not only of patients but of analysts as well.

6.6 RISK CHARACTERIZATION AND PHARMACOPOEIAL IMPURITIES

The results of the risk assessment indicate that from the 57 potentially mutagenic impurities identified, the levels were below the appropriate limit for 54 of them. This shows that although there are potentially mutagenic impurities arising from these drugs, the current manufacturing processes are adequate to eliminate them, reducing their levels and ensuring a negligible risk.

The 3 other impurities are described in a pharmacopoeial monograph with a limit higher than the calculated limit based on ICH M7, hence the limit adopted by the manufacturers comply with the one described in the monograph, and manufacturers were not willing to develop a more sensitive method for those impurities, due to the belief that limits described by the Pharmacopoeia are qualified and considered safe.

This belief may be explained by the fact that pharmacopoeial monographs are references adopted by drug substance and drug product manufacturers, as well as regulatory bodies all over the world, to define the quality criteria which are considered acceptable for drug substances and products. However, the monographs published in these compendia are based on technical and regulatory requirements in force at the time of the preparation of each monograph (EDQM, 2017), so monographs published before ICH M7 came into force reflect specifications considered appropriate when there was no need to investigate the potential of a given impurity being mutagenic.

The question remains as to how limits for these impurities were established for monographs published before ICH M7. The most probable answer is that they were based on ICH Q3A - the guideline for setting limits for impurities in drug substances, which was first published in 1995 - also considering trend results for the impurities in the drug substance. Such limits are not necessarily based on safety studies, since the mutagenic and carcinogenic potential of impurities did not need to be evaluated at the time, neither can they be estimated based on clinical trials which may have supported these applications. Clinical trials enable the identification of side effects related to the use of the drug product, however the development of cancer is a long-term side effect and a multifactorial disease, which should be mitigated during early drug development through the mutagenicity assessment for the drug substance (ICH S1A, 1996) and impurities.

Considering that the mutagenicity assessment of impurities was not performed for most drugs which were approved before ICH M7 came into effect, and that the specifications described in pharmacopoeial monographs are based on requirements in force at the time, this indicates that when there is a mutagenicity alert in the structure of an impurity described in a monograph, it is not appropriate to automatically consider the pharmacopoeial limits as qualified. Even more important than applying the quality aspects defined in the monographs, is for manufacturers to perform their own critical evaluation of the manufacturing process and product, to conclude if all the requirements established in the monograph are in fact applicable for their case and if any additional controls are needed. The recommendation then would be for manufacturers to tighten the limit when necessary, although a revision of the monograph would be a more harmonized approach, which would ensure that all manufacturers comply with the correct limit.

The position of the European Pharmacopoeia regarding revision of already published monographs, as described in EDQM Technical Guide for the Elaboration of Monographs, is that impurity limits can be revised when experimental data is available confirming the mutagenicity of impurities (i.e. class 1 or 2 impurities). It specifically mentions that structural alerts alone would not be enough to trigger a revision of the monograph (i.e. class 3 impurities) (EUROPEAN PHARMACOPOEIA, 2015). This is in line with the current scope of ICH M7, which is also not intended to be applied retrospectively, except when it comes to class 1 and 2 impurities.

Nonetheless, the Lessons Learnt document recently published by EMA regarding nitrosamines recommends for the retrospective application of ICH M7 to be considered (EMA, 2020b). This reiterates the importance of setting appropriate limits and control strategies based on ICH M7 for any drug, regardless of it being on the market or not, which also means that some already approved specifications – and corresponding monographs – may need to be revised.

Moreover, this is in line with the results we obtained, demonstrating that an impurity originally assigned to class 3 was shown to be a class 2 impurity, after conduction of the Ames test, hence indicating the need for a monograph to be revised with the stricter limit. This result also suggests that there may be the need to revise other monographs which list impurities containing mutagenicity alerts with limits higher than would be adequate.

When considering the impacts of limits in a monograph being tightened, there may be a concern of levels being out of specifications. However, this is not expected to be common, since the innate reactivity of these impurities usually ensures their elimination to below the acceptable ICH M7 limits. Nonetheless, in case a class 3 impurity is present above the TTC limit, it may also be possible to justify higher limits if the actual bacterial mutagenicity test is performed yielding a negative result. Even if the impurity is positive in the bacterial mutagenicity test, this can also be further investigated with *in vivo* studies, as described in the guideline (ICH M7 (R1), 2017). However, avoidance of the use of animals in research is encouraged, so when levels of the impurity are below the ICH M7 limit, the recommended approach is not to proceed with *in vivo* testing.

Hence, the greatest impact expected is a change in specifications which are currently in place for drug substances, reducing the limits to appropriate levels depending on impurity classification. Another positive impact in the long term would be for all monographs issued before ICH M7 to be revised, reflecting appropriate limits based on the latest scientific understanding.

7 CONCLUSIONS

The control of mutagenic impurities has been a hot topic in the Pharmaceutical Industry since the recent investigations on ‘sartans’, ranitidine and other drugs which may be contaminated with nitrosamines. However, these carcinogens are not the only mutagenic impurities arising from the routes of synthesis of drug substances.

The results obtained show that in anti-hypertensive drugs, around 22% of the possible impurities are potentially mutagenic. However, the risk assessment showed that their exposure levels are below the acceptable limits, indicating that the current manufacturing processes are adequate to ensure a negligible risk.

Nonetheless, the adherence to ICH M7 requirements regarding the establishment of control strategies for these impurities was lower than expected at the start of the project. Since most anti-hypertensive treatments are well established, many of these manufacturers may not have had the chance to submit their dossiers to new registrations after the publication of ICH M7. Hence it was not a regulatory requirement for them to comply with the guideline. Although all manufacturers were promptly willing to comply with the requirements, the fact that many of them did not have a control strategy in place by the time they were first contacted confirms the hypothesis that it would be appropriate for ICH M7 to be applicable to already approved drug products. This would ensure that control strategies would be proactively applied, ultimately guaranteeing the maintenance of the negligible risk throughout all manufactured batches.

The comparison of *in silico* predictions with experimental results was done for the hazard identification and exposure evaluation, and in both cases the prediction was accurate when compared to the experimental result. The purge factor calculation successfully predicted the level of the impurity determined through an analytical method, and the mutagenicity prediction was correct to consider the impurity as potentially mutagenic. Hence, these two *in silico* approaches were shown to adequately substitute experimental tests, a really positive outcome considering the challenges involved in experimental tests and the advantages of *in silico* tools, which are cheaper, faster, and more sustainable as well.

Finally, the mutagenicity evaluation of pharmacopoeial impurities is encouraged, so that limits can be revised as needed, considering the principles of ICH M7 and the latest scientific understanding.

REFERENCES

AMBERG, A. et al. Principles and procedures for handling out-of-domain and indeterminate results as part of ICH M7 recommended (Q)SAR analyses. **Regulatory Toxicology and Pharmacology**, v. 102, n. December 2018, p. 53–64, 2019.

AMERICAN CANCER SOCIETY. **Known and probable human carcinogens**. Disponível em: <https://www.cancer.org/cancer/cancer-causes/general-info/known-and-probable-human-carcinogens.html>. Acesso em: 5 out. 2018.

AMES, B. N.; LOIS, A.; GOLD, S. Too Many Rodent Carcinogens : Mitogenesis Increases Mutagenesis. **Science**, v. 249, n. 4, p. 970–971, 1990.

ANVISA. **Adoção de medidas para medicamentos para pressão alta**. Disponível em: <http://portal.anvisa.gov.br/noticias/-/asset_publisher/FXrpx9qY7FbU/content/adocao-de-medidas-para-medicamentos-para-pressao-alta/219201>. Acesso em: 13 set. 2019a.

ANVISA. **Relatório de Análise de Impacto Regulatório**. Disponível em: <http://portal.anvisa.gov.br/documents/10181/2961691/Relatório+de+AIR+-+CP+682-2019.pdf/1b59efa8-6065-43ac-8894-9fe7390d4591>. Acesso em: 25 nov. 2019b.

BARBER, C. et al. Establishing best practise in the application of expert review of mutagenicity under ICH M7. **Regulatory Toxicology and Pharmacology**, v. 73, n. 1, p. 367–377, 2015.

BARBER, C. et al. Evaluation of a statistics-based Ames mutagenicity QSAR model and interpretation of the results obtained. **Regulatory Toxicology and Pharmacology**, v. 76, n. 2016, p. 7–20, 2016.

BARBER, C. et al. A consortium-driven framework to guide the implementation of ICH M7 Option 4 control strategies. **Regulatory Toxicology and Pharmacology**, v. 90, p. 22–28, 2017.

BARBER, E. D.; DONISH, W. H.; MUELLER, K. R. A procedure for the quantitative measurement of the mutagenicity of volatile liquids in the Ames Salmonella/microsome assay. **Mutation Research/Genetic Toxicology**, v. 90, n. 1, p. 31–48, 1981.

BERCU, J. P. et al. Potential impurities in drug substances: Compound-specific toxicology limits for 20 synthetic reagents and by-products, and a class-specific toxicology limit for alkyl bromides. **Regulatory Toxicology and Pharmacology**, v. 94, p. 172–182, 1 abr. 2018.

BURNS, M. J. et al. A New Semi-Automated Computer-Based System for Assessing the Purge of Mutagenic Impurities. **Organic Process Research & Development**, 2019.

BURNS, M. J. et al. Controlling a Cohort: Use of Mirabilis-Based Purge Calculations to Understand Nitrosamine-Related Risk and Control Strategy Options. **Organic Process Research and Development**, v. 24, n. 8, p. 1531–1535, 21 ago. 2020.

CMED; ANVISA. **Anuário Estatístico do Mercado Farmacêutico - 2016**. Brasília: [s.n.]. Disponível em: <http://portal.anvisa.gov.br/documents/374947/3413536/Anuário+Estatístico+do+Mercado+Farmacêutico+-+2016/485ddf50-a37f-469f-89e5-29643c5c9df5>.

DELANEY, E. J. An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals. **Regulatory Toxicology and Pharmacology**, v. 49, n. 2, p. 107–124, 2007.

DOBO, K. L. et al. In silico methods combined with expert knowledge rule out mutagenic potential of pharmaceutical impurities: An industry survey. **Regulatory Toxicology and Pharmacology**, v. 62, n. 3, p. 449–455, 2012.

ECHA. **Methyl acetoacetate - Registration Dossier**. Disponível em: <https://echa.europa.eu/registration-dossier/-/registered-dossier/13864/7/7/1>. Acesso em: 29 jan. 2021.

EDQM. Annual Report. p. 39, 2017.

ELDER, D. P.; TEASDALE, A. Is Avoidance of Genotoxic Intermediates/Impurities Tenable for Complex, Multistep Syntheses? **Organic Process Research and Development**, v. 19, n. 11, p. 1437–1446, 2015.

EMA. **Guideline on the Limits of Genotoxic Impurities**, 2006.

EMA. CPMP/ICH/283/95 Impurities: Guideline for residual solvents & CVMP/VICH/502/99 Guideline on impurities: residual solvents. v. 44, n. February, p. 1–6, 2013.

EMA. **EMA reviewing medicines containing valsartan from Zhejiang Huahai following detection of an impurity**. [s.l: s.n.]. Disponível em: <www.ema.europa.eu/contact>.

EMA. EMA advises companies on steps to take to avoid nitrosamines in human medicines (EMA/511347/2019). 2019a.

EMA. **EMA update on metformin diabetes medicines (EMA/660975/2019)**. [s.l: s.n.]. Disponível em: <www.ema.europa.eu/contact>.

EMA. **EMA to review ranitidine medicines following detection of NDMA (EMA/503622/2019)**. [s.l: s.n.]. Disponível em: <www.ema.europa.eu/contact>.

EMA. EMA/217823/2019 Assessment report. v. 31, n. February, p. 1–41, 2019d.

EMA. **EMA/369136/2020 - Assessment report - Procedure under Article 5(3) of Regulation EC (No) 726/2004, Nitrosamine impurities in human medicinal products**. [s.l: s.n.]. Disponível em: <www.ema.europa.eu/contact>.

EMA. Lessons learnt from presence of N-nitrosamine impurities in sartan medicines. **Ema/526934/2019**, 2020b.

ERIKSSON, L. et al. A strategy for ranking environmentally occurring chemicals. Part VI. QSARs for the mutagenic effects of halogenated aliphatics. **Acta Chem Scand**, v. 45, p. 935–944, 1991.

EU. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). 2006.

EUROPEAN COMMISSION. **International Cooperation on Alternative Test Methods (ICATM)**. Disponível em: <https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/advisory-bodies/icatm>. Acesso em: 16 fev. 2021.

EUROPEAN PHARMACOPOEIA. Technical Guide for the Elaboration of Monographs. 2015.

FDA. **Statement from FDA Commissioner Scott Gottlieb, M.D., and Janet Woodcock, M.D., director of the Center for Drug Evaluation and Research on FDA's ongoing investigation into valsartan impurities and recalls and an update on FDA's current findings**. Disponível em: <https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm619024.htm>.

FORD, K. A. Refinement, reduction, and replacement of animal toxicity tests by computational methods. **ILAR Journal**, v. 57, n. 2, p. 226–233, 1 dez. 2016.

GREENE, N. et al. Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR. **SAR and QSAR in environmental research**, v. 10, n. 2–3, p. 299–314, 1999.

HASSELGREN, C. et al. Management of pharmaceutical ICH M7 (Q)SAR predictions – The impact of model updates. **Regulatory Toxicology and Pharmacology**, v. 118, 1 dez. 2020.

HAYES, A. W.; KRUGER, C. L. **Hayes' Principles and Methods of Toxicology**. 6. ed. [s.l.] CRC Press, 2014.

HOLLEMAN, A. F.; WIBERG, E., WIBERG, N. **Inorganic Chemistry translated by Eagleson, Mary; Brewer, William**. San Diego/Berlin: [s.n.].

HONMA, M. et al. Improvement of quantitative structure-activity relationship (QSAR) tools for predicting Ames mutagenicity: Outcomes of the Ames/QSAR International Challenge Project. **Mutagenesis**, v. 34, n. 1, p. 41–48, 2019.

ICH M3 (R2). Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. v. 3, n. July, 2008.

ICH M4Q (R1). The Common Technical Document for the Registration of Pharmaceuticals for Human Use: Quality. **Drugs Made in Germany**, v. 35, n. 1, p. 12–24, 1992.

ICH M7 Q&A. **ICH guideline M7 on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk - questions & answers - Step 2b**. [s.l: s.n.]. Disponível em: <http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono>.

ICH M7 (R1). **Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk**, 2017.

ICH Q3A (R2). **Impurities in New Drug Substances**, 2006.

ICH Q3B (R2). **Impurities in New Drug Products**, 2006.

ICH Q3C (R6). **Impurities: Guideline for Residual Solvents**, 2016.

ICH Q3D (R1). **Guideline for Elemental Impurities**. [s.l: s.n.].

ICH Q11. Development and manufacture of drug substances (chemical entities and biotechnological/ biological entities). v. 44, n. May, 2012.

ICH S1A. The Need for Carcinogenicity Studies of Pharmaceuticals. n. December 1995, p. 1–5, 1996.

JAPAN NATIONAL INSTITUTE OF HEALTH SCIENCES. **Methyl acetoacetate**. Disponível em: <https://dra4.nihs.go.jp/mhlw_data/home/file/file105-45-3.html#:~:text=Methyl%20acetoacetate%20was%20studied%20for,mg%2Fkg%20for%20both%20sexes>. Acesso em: 29 jan. 2021.

JIA YULIANG ZHANG FUCHENG. **CN105129752A**China, 2015. Disponível em: <https://patents.google.com/patent/CN105129752A/en>

KROES, R. et al. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. **Food Chem Toxicol**, v. 42, p. 65–83, 2004.

LHASA LIMITED. **Lhasa Limited**. Disponível em: <https://www.lhasalimited.org/>. Acesso em: 6 out. 2018.

LHASA LIMITED. **Carcinogenicity Database**. Disponível em: <https://carcdb.lhasalimited.org/carcdb-frontend/>. Acesso em: 16 nov. 2019.

LÜLLMANN, H. M. K. H. L. B. D. **Farmacologia: texto e atlas**. [s.l: s.n.].

MENGUE, S. S. et al. Access to and use of high blood pressure medications in Brazil. **Revista de Saude Publica**, v. 50, n. supl 2, p. 1–9, 2016.

MÜLLER, L. et al. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. **Regulatory Toxicology and Pharmacology**, v. 44, n. 3, p. 198–211, 2006.

MYATT, G. J. et al. In silico toxicology protocols. **Regulatory Toxicology and Pharmacology**, v. 96, n. April, p. 1–17, 2018.

NRC. Appendix C: Overview of Risk Assessment. In: NATIONAL RESEARCH COUNCIL (US) COMMITTEE ON APPLICATIONS OF TOXICOGENOMIC TECHNOLOGIES TO PREDICTIVE TOXICOLOGY. **Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment**. Washington, DC: National Academies Press, 2007.

NTP. **Tox21: Toxicology Testing in the 21st Century**. Disponível em: <https://www.niehs.nih.gov/health/materials/tox21_508.pdf>. Acesso em: 16 fev. 2021.

OECD. OECD Guideline for the Testing of Chemicals OECD 451. n. July, p. 1–17, 2011.

OECD. **Test Guideline No. 471 Bacterial Reverse Mutation Test Section 4 Health effects OECD/OCDE 471 OECD GUIDELINE FOR TESTING OF CHEMICALS Bacterial Reverse Mutation Test**. [s.l: s.n.].

PECORINO, L. DNA structure and stability: mutations versus repair. In: OXFORD UNIVERSITY PRESS (Ed.). . **Molecular Biology of Cancer**. 3. ed. [s.l: s.n.]. p. 21–51.

PLOŠNIK, A.; VRAČKO, M.; DOLENC, M. S. Mutagenic and carcinogenic structural alerts and their mechanisms of action. **Arhiv za Higijenu Rada i Toksikologiju**, v. 67, n. 3, p. 169–182, 2016.

ROSTKOWSKA, K. et al. Formation and metabolism of N-nitrosamines. **Polish Journal of Environmental Studies**, v. 7, n. 6, p. 321–325, 1998.

RUSSELL, W. M. S.; BURCH, R. L. **The Principles of Humane Experimental Technique**. London: [s.n.].

SHIMIZU, H. et al. The results of microbial mutation test for forty-three industrial chemicals. **Japanese Journal of Industrial Health**, 1985.

SPEAKING OF RESEARCH. **US Statistics**. Disponível em: <https://speakingofresearch.com/facts/statistics/>. Acesso em: 5 out. 2018.

STANTON, K.; KRUSZEWSKI, F. H. Quantifying the benefits of using read-across and in silico techniques to fulfill hazard data requirements for chemical categories. **Regulatory Toxicology and Pharmacology**, v. 81, p. 250–259, 1 nov. 2016.

STATISTA. **Animals used in research experiments worldwide - 2016**. Disponível em: <https://www.statista.com/statistics/639954/animals-used-in-research-experiments-worldwide/>. Acesso em: 6 out. 2018.

SUTTER, A. et al. Use of in silico systems and expert knowledge for structure-based assessment of potentially mutagenic impurities. **Regulatory Toxicology and Pharmacology**, v. 67, n. 1, p. 39–52, 2013.

TAYLOR, K. et al. Estimates for worldwide laboratory animal use in 2005. **ATLA Alternatives to Laboratory Animals**, v. 36, n. 3, p. 327–342, 2008.

TEASDALE, A. et al. A Tool for the Semiquantitative Assessment of Potentially Genotoxic Impurity (PGI) Carryover into API Using Physicochemical Parameters and Process Conditions. **Organic Process Research & Development**, v. 14, n. 4, p. 943–945, 2010.

TEASDALE, A. et al. Risk assessment of genotoxic impurities in new chemical entities: Strategies to demonstrate control. **Organic Process Research and Development**, v. 17, n. 2, p. 221–230, 2013.

US EPA. **Memorandum: Directive to Prioritize Efforts to Reduce Animal Testing**. Disponível em: <https://www.epa.gov/sites/production/files/2019-09/image2019-09-09-231249.txt>. Acesso em: 16 fev. 2021.

VALERIO, L. G. In silico toxicology for the pharmaceutical sciences. **Toxicology and Applied Pharmacology**, v. 241, n. 3, p. 356–370, 2009.

WILLIAMS, R. V. et al. It's difficult, but important, to make negative predictions. **Regulatory Toxicology and Pharmacology**, v. 76, p. 79–86, 2016.

WILLIAMS, R. V. et al. Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed? **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 848, n. August, p. 503081, 2019.

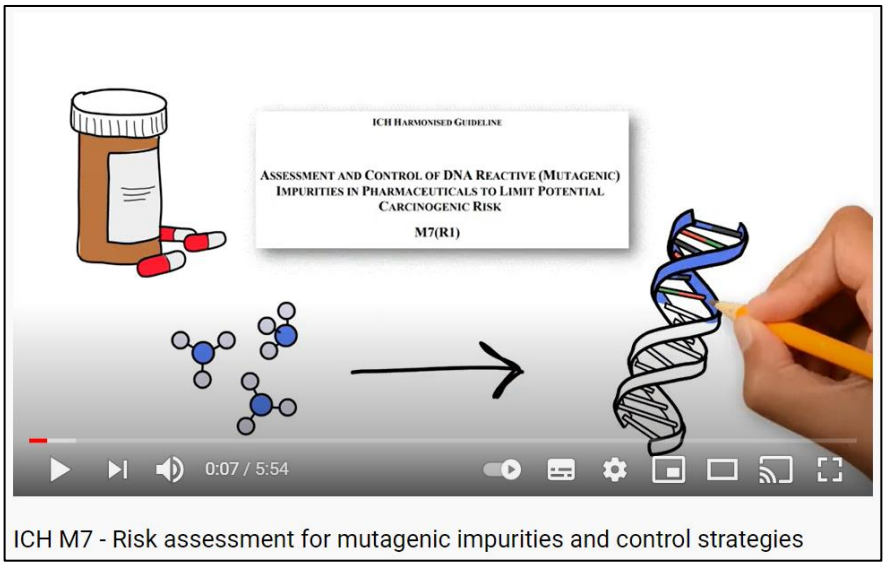
ANNEX

Annex A - Videos

The following videos were made using VideoScribe, with the aim to turn ICH M7 concepts and the purge factor calculation into something easy and accessible to drug substance manufacturers, regulators, colleagues across industry, the scientific community, and others who may be interested.

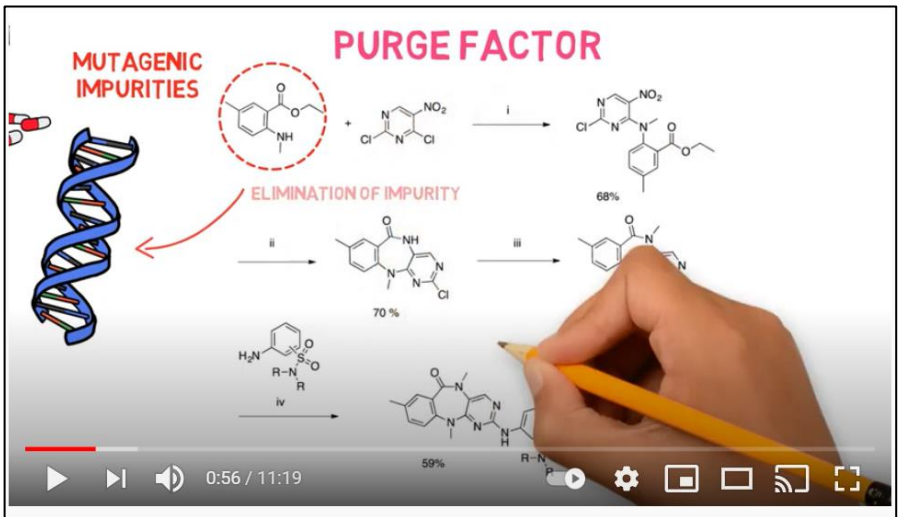
They are publicly available on YouTube at the following links:

"**ICH M7 - Risk assessment for mutagenic impurities and control strategies**" – available at https://youtu.be/B9XahAHOdAw



ICH M7 - Risk assessment for mutagenic impurities and control strategies

"**The purge factor - ICH M7 Option 4**" – available at https://youtu.be/OnclED3e4jc



The purge factor - ICH M7 Option 4

Annex B – Student transcript (Ficha do aluno)

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
FICHA DO ALUNO

9143 - 11094837/1 - Fernanda Waechter

Email:	fernanda.waechter@usp.br
Data de Nascimento:	02/11/1989
Cédula de Identidade:	RG - 11.053.470.64 - RS
Local de Nascimento:	Estado do Rio Grande do Sul
Nacionalidade:	Brasileira
Graduação:	Farmacêutica - Universidade Federal do Rio Grande do Sul - Brasil - 2015

Curso:	Mestrado
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Toxicologia
Data de Matrícula:	14/01/2019
Início da Contagem de Prazo:	14/01/2019
Data Limite para o Depósito:	14/07/2021
Orientador Acadêmico:	Prof(a). Dr(a). Sandro Rogerio de Almeida - 14/01/2019 até 12/03/2019. Email: sandroal@usp.br
Orientador:	Prof(a). Dr(a). Elizabeth de Souza Nascimento - 13/03/2019 até o presente. Email: esnasci@usp.br
Co-orientador:	Prof(a). Dr(a). Antonio Anáx Falcão de Oliveira - 11/09/2019 até o presente. Email: antonioanax@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 14/01/2019
Data de Aprovação no Exame de Qualificação:	Aprovado em 06/03/2020
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 14/01/2019

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).
Última ocorrência: Matrícula Regular em 27/07/2020
Impresso em: 17/02/2021 10:29:30

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
FICHA DO ALUNO

9143 - 11094837/1 - Fernanda Waechter

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBF5756-5/2	Segurança Biológica de Produtos Farmacêuticos, Biotecnológicos e Biomateriais	11/03/2019	14/04/2019	90	6	100	A	N	Concluída
FBC5793-14/2	Tópicos em Fisiopatologia e Toxicologia I	12/03/2019	24/06/2019	15	1	100	A	N	Concluída
FBC5729-8/2	Fundamentos Básicos da Avaliação do Risco Oferecido por Substâncias Químicas	08/04/2019	05/05/2019	60	4	100	A	N	Concluída
FBC5757-8/3	Tópicos em Fisiopatologia e Toxicologia II	06/08/2019	18/11/2019	15	1	100	A	N	Concluída
QFL5933-12/3	Fundamentos da Química Orgânica (Instituto de Química - Universidade de São Paulo)	17/03/2020	29/06/2020	150	10	100	A	N	Concluída
RBP5792-1/2	Mecanismos Moleculares do Câncer (Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo)	28/09/2020	18/10/2020	45	3	100	A	N	Concluída
8055740-2/3	Scientific Publication: Manuscript Submission and Peer Review Process (Faculdade de Odontologia de Ribeirão Preto - Universidade de São Paulo)	12/11/2020	16/12/2020	60	0	-	-	N	Matrícula cancelada

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito da dissertação	
Disciplinas:	0	25	25
Estágios:			
Total:	0	25	25

Créditos Atribuídos à Dissertação: 71

Conceito a partir de 02/01/1997:
A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.
Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula Regular em 27/07/2020
Impresso em: 17/02/2021 10:29:30

Annex C – Lattes CV (Currículo Lattes)

Currículo Lattes

Imprimir currículo

Fernanda Waechter

Endereço para acessar este CV: **http://lattes.cnpq.br/1960543594435594**

Última atualização do currículo em 20/02/2021

Resumo informado pelo autor

Farmacêutica, graduada pela Universidade Federal do Rio Grande do Sul. Atualmente mestranda no Programa de Pós-Graduação em Farmácia (Fisiopatologia e Toxicologia) na Faculdade de Ciências Farmacêuticas da USP, e pesquisadora no Aché Laboratórios, com foco em avaliação do risco de impurezas mutagênicas (ICH M7) e nitrosaminas.

(Texto informado pelo autor)

Nome civil

Nome Fernanda Waechter

Dados pessoais

Filiação Rolf Edison Waechter e Rose Isabel Waechter

Nascimento 02/11/1989 - Brasil

Carteira de Identidade 1105347064 SJS - RS - 04/10/2005

CPF 026.419.040-80

Formação acadêmica/titulação

2019 Mestrado em Farmácia (Fisiopatologia e Toxicologia).
Faculdade de Ciências Farmacêuticas - USP, FCF-USP, Brasil
Título: Avaliação do risco de impurezas potencialmente mutagênicas em medicamentos registrados no Brasil
Orientador: Elizabeth de Souza Nascimento
Co-orientador: Antonio Anax de Oliveira Falcão

2009 - 2015 Graduação em Farmácia.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
com **período sanduíche** em University of California, Irvine (Orientador: Debbie Wong)
Título: Design, synthesis, and biological evaluation of betulinic acid derivatives as new antitumor agents
Orientador: Simone Cristina Baggio Gnoatto
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Graduação interrompido(a) em Biotecnologia.
Pitt Community College, PCC, Estados Unidos
Ano de interrupção: 2008

2005 - 2007 Ensino Médio (2o grau) .
Colégio Mauá, CM, Brasil

Formação complementar

2020 - 2020 Curso de curta duração em Mutagenic impurities - the impact of N-Nitrosamines. (Carga horária: 6h).
Scientific Update, SCIENTIFIC, Inglaterra

2019 - 2019 Curso de curta duração em Avaliação de DIFA sob a ótica do novo marco regulatório de IFAs no Brasil. (Carga horária: 16h).
Unite Consultoria, Treinamentos e Eventos, UNITE, Brasil

2016 - 2016 Curso de curta duração em D001x: Medicinal Chemistry: The Molecular Basis of Drug Discovery.
Davidson College, D.C, Davidsn, Estados Unidos

2016 - 2016 Curso de curta duração em Avaliação de DMF - IFA. (Carga horária: 16h).
Unite Consultoria, Treinamentos e Eventos, UNITE, Brasil

2015 - 2015 Curso de curta duração em Curso Análise Instrumental - HPLC. (Carga horária: 20h).
Prati-Donaduzzi, PRATI-DONADUZZI, Toledo, Brasil

2014 - 2014 Curso de curta duração em Desenvoltura em Oratória e Comunicação (DOC). (Carga horária: 20h).
Humanize, HUMANIZE, Brasil

2013 - 2013 Curso de curta duração em Human Subject Research Course. (Carga horária: 5h).
Collaborative Institutional Training Initiative, CITI, Estados Unidos

2012 - 2012 Curso de curta duração em Desenvolvimento da inteligência emocional. (Carga horária: 30h).
Conexão Alpha Treinamentos, CONEXÃO ALPHA, Brasil

2012 - 2012 Curso de curta duração em Lab Animal Training. (Carga horária: 5h).
Collaborative Institutional Training Initiative, CITI, Estados Unidos

2010 - 2010 Curso de curta duração em Operação e manutenção básica do Sistema HPLC. (Carga horária: 16h).
Scientific Instruments Co., SINC, Brasil

2010 - 2010 Curso de curta duração em Inovação em Cosméticos. (Carga horária: 16h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2009 - 2009 Curso de curta duração em Interação Medicamentos e Alimentos. (Carga horária: 10h).
Associação dos Farmacêuticos do Rio Grande do Sul, AFARGS, Brasil

2009 - 2009 Curso de curta duração em Estresse Oxidativo: patologias relacionadas. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2009 - 2009 Curso de curta duração em Conceitos e Aplicações Farmacêuticas da Nanotecnologia. (Carga horária: 14h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2006 - 2006 Curso de curta duração em Comprehensive English Program at Level 5. (Carga horária: 80h).
GEOS Language Academy - Ottawa, GEOS, Canadá

Atuação profissional

1. Aché Laboratórios Farmacêuticos - ACHÉ

Vínculo institucional

2017 - Atual Vínculo: Celetista , Enquadramento funcional: Especialista de DMF , Carga horária: 40, Regime: Dedicação exclusiva

2. Prati, Donaduzzi & Cia Ltda. - PRATI-DONADUZZI

Vínculo institucional

2015 - 2017 Vínculo: Celetista , Enquadramento funcional: Analista de Assuntos Regulatórios , Carga horária: 44, Regime: Dedicação exclusiva
Outras informações:
Analista de Assuntos Regulatórios com ênfase em análise de DMF.

2014 - 2014 Vínculo: estagiário , Enquadramento funcional: estagiário , Carga horária: 30, Regime: Dedicação exclusiva
Outras informações:
Programa de estágio Sementes para o Futuro - Gestão da Indústria Farmacêutica.

3. University of California, Irvine - UCI

Vínculo institucional

2013 - 2013

4. Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul - FAPERGS

Vínculo institucional

2012 - 2012

5. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2013 - 2013 Vínculo: Bolsista , Enquadramento funcional: Bolsista de Extensão , Carga horária: 20, Regime: Parcial

2010 - 2011 Vínculo: Estagiária , Enquadramento funcional: Bolsista de iniciação científica , Carga horária: 20, Regime: Parcial

6. Agência Nacional de Vigilância Sanitária - ANVISA

Vínculo institucional

2020 - Atual

7. Universidade de São Paulo - USP

Vínculo institucional

2019 - Atual

Projetos

Projetos de pesquisa

2019 - Atual Avaliação do risco de impurezas potencialmente mutagênicas em medicamentos registrados no Brasil

Descrição: A presença de impurezas mutagênicas em medicamentos tem sido controlada conforme normas internacionais no Brasil e no mundo (ICH M7). O ICH M7 não é aplicável a medicamentos já aprovados, porém recentemente impurezas mutagênicas foram encontradas acima dos limites permitidos na valsartana, um medicamento já aprovado por agências reguladoras. O objetivo desse trabalho é identificar as possíveis impurezas mutagênicas em medicamentos anti-hipertensivos registrados no Brasil, e fazer a avaliação do risco propondo estratégias de controle para tais impurezas.
Situação: Em andamento Natureza: Projetos de pesquisa
Alunos envolvidos: Mestrado acadêmico (1);
Integrantes: Fernanda Waechter; Elizabeth de Souza Nascimento (Responsável); Antonio Anax Falcão de Oliveira
Número de produções C,T & A: 1/

2014 - 2014 Obtenção de compostos naturais e sintéticos com potenciais efeitos protetivos ou deletérios para a saúde humana visando o planejamento de novos candidatos a fármacos

Situação: Concluído Natureza: Projetos de pesquisa
Integrantes: Fernanda Waechter; Gloria Santos; Simone Gnoatto (Responsável)

2013 - 2013 Determinação de Componentes do Chá Verde em Moscas de Fruta

Descrição: O projeto teve como objetivo desenvolver um método de extração e identificação por HPLC, de catequinas - os principais componentes do chá verde - presentes em moscas Drosophila. As moscas foram alimentadas inicialmente com comida tradicional para moscas, e mais tarde por meio de solução de sacarose em um capilar. A cada forma de alimentação foi-se adicionado chá verde ou não (controle). Após foi feita uma comparação das amostras extraídas de moscas que foram alimentadas com chá verde com as controle, relacionando com o cromatograma do extrato de chá verde.
Situação: Concluído Natureza: Projetos de pesquisa
Alunos envolvidos: Graduação (1); Especialização (1);
Integrantes: Fernanda Waechter; Mahtab Jafari (Responsável); Terry Lopez

2010 - 2011 AVALIAÇÃO DA DEFICIÊNCIA MICRONUTRICIONAL EM IDOSOS, SEUS RISCOS PATOLÓGICOS E PREVENÇÃO

Situação: Concluído Natureza: Projetos de pesquisa
Integrantes: Fernanda Waechter; Baierle, Marília; Solange Cristina Garcia (Responsável)
Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq

2009 - 2010 Exposição de indivíduos a xenobióticos e seus efeitos toxicológicos

Descrição: O ser humano está constantemente exposto a diferentes xenobióticos, seja no âmbito laboral ou ambiental. Por outro lado, sabe-se que além dos efeitos diretos destes agentes sobre a saúde humana há a variabilidade individual como predisposição genética e hábitos de vida. Neste sentido, pretende-se estudar, através de biomarcadores toxicológicos e bioquímicos, os efeitos de xenobióticos sobre a saúde humana.
Situação: Concluído Natureza: Projetos de pesquisa
Integrantes: Fernanda Waechter; Moro, Angela M.; Charão, Mariele; Brucker, Natália; Bulcão, Rachel; Freitas, Fernando; Juliana Valentini; Solange Cristina Garcia (Responsável); Limberger, Renata P; Thiessen, Flavia V; Leal, Mirna B

Projetos de desenvolvimento tecnológico

2012 - 2012 Aplicação da modelagem molecular no planejamento racional de novos candidatos a protótipos de agentes anticoagulantes e antitrombóticos

Situação: Desativado Natureza: Projetos de desenvolvimento tecnológico
Integrantes: Fernanda Waechter; Hugo Verli (Responsável)
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

Projeto de extensão

2013 - 2013 Prestação de informação sobre medicamentos a profissionais da saúde pelo CIM-RS

Descrição: O Centro de Informações sobre Medicamentos do Rio Grande do Sul (CIM-RS), uma parceria da UFRGS com o Conselho Regional de Farmácia do RS, caracteriza-se como uma fonte de informação técnico-científica sobre medicamentos para os profissionais da saúde. O principal objetivo do CIM-RS é assessorar, de forma imparcial, profissionais da saúde de nível superior em assuntos relacionados a medicamentos, colaborando para seu uso seguro e racional.
Situação: Concluído Natureza: Projeto de extensão
Integrantes: Fernanda Waechter (Responsável); ; Tatiane da Silva Dal-Pizzol; Clarissa Ruaro Xavier; Juliana Petry
Financiador(es): Universidade Federal do Rio Grande do Sul-UFRGS

2010 - 2010 BIOMARCADORES DE EXPOSIÇÃO PARA O ACOMPANHAMENTO DE INDIVÍDUOS OCUPACIONALMENTE EXPOSTOS AOS AGENTES QUÍMICOS

Situação: Concluído Natureza: Projeto de extensão
Integrantes: Fernanda Waechter; Solange Cristina Garcia (Responsável)
Financiador(es): Universidade Federal do Rio Grande do Sul-UFRGS

Outros tipos de projetos

2020 - Atual Grupo de Trabalho - Controle de Nitrosaminas em Medicamentos

Descrição: O Grupo de Trabalho foi instituído pela Portaria nº 630, de 9 de outubro de 2020, no âmbito da Agência Nacional de Vigilância Sanitária e outras associações parceiras, para discussão técnica sobre o controle de nitrosaminas em medicamentos e proposição de Processo Regulamentar.
Situação: Em andamento Natureza: Outros tipos de projetos
Integrantes: Fernanda Waechter (Responsável); ; Antonio Anax Falcão de Oliveira; Alana Silva da Purificação Galeno; Rosimeire Pereira Alves da Cruz; Maria Augusta Carvalho Rodrigues; Nayrton Flávio Moura Rocha; Cejana Brasil Cirilo Passos; Elizabete Regina Viana Freitas; Edvaldo Oliveira Neves; Marcos Matos Lima; Jasquer Sehnem

Áreas de atuação

1. Farmácia

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol Compreende Razoavelmente , Fala Pouco , Escreve Pouco , Lê Razoavelmente

Francês Compreende Razoavelmente , Fala Razoavelmente , Escreve Razoavelmente , Lê Bem

Português Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Prêmios e títulos

2015 Mérito Acadêmico, Conselho Regional de Farmácia do Rio Grande do Sul

2014 Destaque na sessão Farmácia - Química Medicinal e Farmacologia do XXVI SIC, Universidade Federal do Rio Grande do Sul

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. **Waechter, Fernanda**; DA SILVA, GLORIA N.S.; WILLIG, JULIA B.; DE OLIVEIRA, CRISTIANE B.; VIEIRA, BRUNA D.; TRIVELLA, DANIELA B.B.; ZIMMER, ALINE R.; BUFFON, ANDREIA; PILGER, DIOGO A.; GNOATTO, SIMONE C.B.
Design, Synthesis and Biological Evaluation of Betulinic Acid Derivatives as New Antitumor Agents for Leukemia. Anti-Cancer Agents in Medicinal Chemistry. JCR, v.17, p.1 - 9, 2018.
Referências adicionais: Inglês. Meio de divulgação: Meio digital

2. Moro, Angela M.; Charão, Mariele; Brucker, Natália; Bulcão, Rachel; Freitas, Fernando; Guerreiro, Gilian; Baierle, Marília; Nascimento, Sabrina; WAECHTER, F.; Hirakata, Vânia
Effects of low-level exposure to xenobiotics present in paints on oxidative stress in workers. Science of the Total Environment. JCR, v.408, p.4461 - 4467, 2010.
Referências adicionais: Inglês. Meio de divulgação: Meio digital. Home page: [doi:10.1016/j.scitotenv.2010.06.058]

Trabalhos publicados em anais de eventos (resumo)

1. Bulcão, Rachel; Baierle, Marília; Moro, Angela M.; Freitas, Fernando; **WAECHTER, F.**; Nascimento, Sabrina; GARCIA, S. C.
Biomarcadores do estresse oxidativo na exposição ao etilbenzeno e ao estireno. In: XX Congresso Pan-Americano de Farmácia e XIV Congresso da Federação Farmacêutica Sul-Americana, 2010, Porto Alegre-RS.
Anais do XX Congresso Pan-Americano de Farmácia e XIV Congresso da Federação Farmacêutica Sul-Americana. , 2010.
Referências adicionais: Brasil/Português.

2. **WAECHTER, F.**; VALENTINI, J.; Barbosa JR F; Passos CJ; GARCIA, S. C.
Exposição ao mercúrio em comunidades do Rio Tapajós, Amazônia, e sua associação com os níveis de GSH In: III Congresso Brasileiro de Toxicologia Clínica, 2010, Florianópolis.
Anais do III Congresso Brasileiro de Toxicologia Clínica. , 2010.
Referências adicionais: Brasil/Português. Meio de divulgação: Impresso

Apresentação de trabalho e palestra

1. **WAECHTER, F.**
Controle de impurezas mutagênicas: uso da opção 4 do ICH M7 no gerenciamento de risco de N-nitrosaminas, 2020. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Virtual; Evento: Tendências e diretrizes para avaliação e controle de impurezas mutagênicas em medicamentos; Inst.promotora/financiadora: Altox

2. **WAECHTER, F.**
Nitrosamines - The Experience of Aché, 2020. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Virtual; Evento: Webinar on Nitrosamines: Cases Studies and Control Strategies; Inst.promotora/financiadora: Sindusfarma

3. **WAECHTER, F.**
Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil, 2020. (Simpósio,Apresentação de Trabalho)
Referências adicionais: Brasil/Português; Local: Virtual; Evento: 3rd World Summit on Toxicology & Applied Pharmacology; Inst.promotora/financiadora: Scientific Federation

4. **WAECHTER, F.**
Risk assessment of potentially mutagenic impurities in drug products, 2020. (Congresso,Apresentação de Trabalho)
Referências adicionais: Brasil/Português; Local: Virtual; Evento: 8º COSIMP - Congresso de Ciências Farmacêuticas do Mercosul - 8º Simpósio em Ciência e Tecnologia de Alimentos do Mercosul; Inst.promotora/financiadora: Unioeste

5. **WAECHTER, F.**; MEIRELLES, L.; Durgante, Juliano; AMADOR, T.
Cuidados com Medicamentos, 2014. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Bretão. Meio de divulgação: Meio digital; Local: Centro Municipal de Educação do Trabalhador Paulo Freire (CMET); Cidade: Porto Alegre; Evento: Palestra sobre uso racional e descarte de medicamentos; Inst.promotora/financiadora: UFRGS

6. **WAECHTER, F.**; SANTOS, G.; GNOATTO, S.
Semi-síntese e avaliação da atividade antitumoral de derivados do ácido betulínico, 2014. (Outra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Vários; Local: Campus do Vale - UFRGS; Cidade: Porto Alegre; Evento: XXVI Salão de Iniciação Científica; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

7. **WAECHTER, F.**; RODRIGUES, P.L.; STAUDT, A.F.; MICHELS, M.S.; NASCIMENTO, J.
Workshop Ciência sem Fronteiras, 2014. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Faculdade de Farmácia - UFRGS; Cidade: Porto Alegre; Evento: 40º Semana Acadêmia de Estudos Farmacêuticos; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

8. **WAECHTER, F.**; PETRY, J.; DAL-PIZZOL, T. S.
Caracterização das atividades desenvolvidas no CIM-RS, 2013. (Outra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Universidade Federal do Rio Grande do Sul; Cidade: PORTO ALEGRE; Evento: XIV Salão de Extensão; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

9. **WAECHTER, F.**; Charão, Mariele; Moro, Angela M.; Brucker, Natália; Baierle, Marília; Freitas, Fernando; Nascimento, Sabrina; Guerreiro, Gilian; Durgante, Juliano; Gauer, Bruna; VALENTINI, J.; GARCIA, S. C.
Avaliação do sistema antioxidante e peroxidação lipídica em trabalhadores expostos a tintas, 2011. (Congresso,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Impresso; Cidade: Novo Hamburgo; Evento: IV Congresso Internacional de Bioanálises, VII Congresso Sul-Brasileiro de Biomedicina e XI Semana Gaúcha de Biomedicina; Inst.promotora/financiadora: Feevale - Instituto de Ciências da Saúde - ICS - Curso de Biomedicina

10. **WAECHTER, F.**; Charão, Mariele; Baierle, Marília; GARCIA, S. C.
Validação de metodologia para quantificação de vitaminas lipossolúveis, importantes antioxidantes na saúde de idosos, 2011. (Outra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Vários; Local: Universidade Federal do Rio Grande do Sul - Campus Centro; Cidade: Porto Alegre; Evento: SIC2011 - XXII Salão de Iniciação Científica da UFRGS; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

11. **WAECHTER, F.**; VALENTINI, J.; Barbosa JR F; Passos CJ; GARCIA, S. C.
Exposição ao mercúrio em comunidades do Rio Tapajós, Amazônia, e sua associação com os níveis de GSH, 2010. (Congresso,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Impresso; Evento: III Congresso Brasileiro de Toxicologia Clínica

12. **WAECHTER, F.**; Durgante, Juliano; Moro, Angela M.; GARCIA, S. C.
Níveis urinários dos biomarcadores de exposição em trabalhadores expostos a tintas, 2010. (Outra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Universidade Federal do Rio Grande do Sul - Campus Centro; Cidade: Porto Alegre; Evento: XI Salão de Extensão; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

Produção técnica

Demais produções técnicas

1. SILVA JUNIOR, P. E.; **WAECHTER, F.**; BUCHALA, A.
Controle de nitrosaminas em medicamentos, 2020. (Outro, Curso de curta duração ministrado)
Referências adicionais: Brasil/Português. 8 horas. Meio de divulgação: Meio digital

2. **WAECHTER, F.**
ICH M7 - Risk assessment for mutagenic impurities and control strategies, 2019. (Desenvolvimento de material didático ou instrucional)
Áreas do conhecimento: Farmácia,Toxicologia
Referências adicionais: Brasil/Inglês. Meio de divulgação: Meio digital. Home page: https://www.youtube.com/watch?v=B9XahAHOdAw&feature=youtu.be
This video explains the principles outlined in ICH M7 and how we should apply this guideline to control mutagenic impurities in APIs.

Inovação

Projetos

Projetos de desenvolvimento tecnológico

2012 - 2012 Aplicação da modelagem molecular no planejamento racional de novos candidatos a protótipos de agentes anticoagulantes e antitrombóticos

Situação: Desativado Natureza: Projetos de desenvolvimento tecnológico
Integrantes: Fernanda Waechter; Hugo Verli (Responsável)
Financiador(es): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul-FAPERGS

Educação e Popularização de C&T

Apresentação de trabalho e palestra

1. **WAECHTER, F.**; MEIRELLES, L.; Durgante, Juliano; AMADOR, T.
Cuidados com Medicamentos, 2014. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Bretão. Meio de divulgação: Meio digital; Local: Centro Municipal de Educação do Trabalhador Paulo Freire (CMET); Cidade: Porto Alegre; Evento: Palestra sobre uso racional e descarte de medicamentos; Inst.promotora/financiadora: UFRGS

2. **WAECHTER, F.**; RODRIGUES, P.L.; STAUDT, A.F.; MICHELS, M.S.; NASCIMENTO, J.
Workshop Ciência sem Fronteiras, 2014. (Conferência ou palestra,Apresentação de Trabalho)
Referências adicionais: Brasil/Português. Meio de divulgação: Meio digital; Local: Faculdade de Farmácia - UFRGS; Cidade: Porto Alegre; Evento: 40º Semana Acadêmia de Estudos Farmacêuticos; Inst.promotora/financiadora: Universidade Federal do Rio Grande do Sul

Desenvolvimento de material didático ou instrucional

1. **WAECHTER, F.**
ICH M7 - Risk assessment for mutagenic impurities and control strategies, 2019. (Desenvolvimento de material didático ou instrucional)
Áreas do conhecimento: Farmácia,Toxicologia
Referências adicionais: Brasil/Inglês. Meio de divulgação: Meio digital. Home page: https://www.youtube.com/watch?v=B9XahAHOdAw&feature=youtu.be
This video explains the principles outlined in ICH M7 and how we should apply this guideline to control mutagenic impurities in APIs.

Demais produções técnicas

1. **WAECHTER, F.**
ICH M7 - Risk assessment for mutagenic impurities and control strategies, 2019. (Desenvolvimento de material didático ou instrucional)
Áreas do conhecimento: Farmácia,Toxicologia
Referências adicionais: Brasil/Inglês. Meio de divulgação: Meio digital. Home page: https://www.youtube.com/watch?v=B9XahAHOdAw&feature=youtu.be
This video explains the principles outlined in ICH M7 and how we should apply this guideline to control mutagenic impurities in APIs.

Eventos

Eventos

Participação em eventos

1. **3rd World Summit on Toxicology & Applied Pharmacology**, 2020. (Congresso)
Risk assessment of potentially mutagenic impurities in anti-hypertensive drug products approved in Brazil.

2. **4th Annual Impurities: Genotoxic & Beyond Virtual Summit**, 2020. (Simpósio)
.

3. Apresentação Oral no(a) **8º COSIMP - Congresso de Ciências Farmacêuticas do Mercosul - 8º Simpósio em Ciência e Tecnologia de Alimentos do Mercosul**, 2020. (Congresso)
Risk assessment of potentially mutagenic impurities in drug products.

4. **ECA - Nitrosamine Impurities**, 2020. (Simpósio)
.

5. **Nitrosamine Impurities Forum**, 2020. (Simpósio)
.

6. **2019 Pharmaceutical Industry and Regulators Symposium**, 2019. (Simpósio)
.

7. **Métodos Alternativos ao uso de animais em pesquisa reconhecidos no Brasil**, 2019. (Oficina)
.

1. Simposista no(a) **40º Semana Acadêmia de Estudos Farmacêuticos**, 2014. (Oficina)
Workshop Ciência sem Fronteiras.

2. **XXVI Salão de Iniciação Científica**, 2014. (Outra)
Semi-síntese e avaliação da atividade antitumoral de derivados do ácido betulínico.

3. **XIV Salão de Extensão**, 2013. (Outra)
Caracterização das atividades desenvolvidas no CIM-RS.

4. **II Congresso Sul de Toxicologia Clínico-Laboratorial**, 2011. (Congresso)
.

5. Apresentação de Poster / Painel no(a) **IV Congresso Internacional de Bioanálises, VII Congresso Sul-Brasileiro de Biomedicina e XI Semana Gaúcha de Biomedicina**, 2011. (Congresso)
Avaliação do sistema antioxidante e peroxidação lipídica em trabalhadores expostos a tintas.

6. Apresentação Oral no(a) **SIC2011 - XXII Salão de Iniciação Científica da UFRGS**, 2011. (Outra)
Validação de metodologia para quantificação de vitaminas lipossolúveis, importantes antioxidantes na saúde de idosos.

7. **XXXVII Semana Acadêmica de Estudos Farmacêuticos**, 2011. (Outra)
.

8. **33º Encontro Nacional dos Estudantes de Farmácia**, 2010. (Encontro)
.

9. Apresentação de Poster / Painel no(a) **III Congresso Brasileiro de Toxicologia Clínica**, 2010. (Congresso)
Exposição ao mercúrio em comunidades do Rio Tapajós, Amazônia, e sua associação com os níveis de GSH.

10. Apresentação Oral no(a) **XI Salão de Extensão**, 2010. (Outra)
Níveis urinários dos biomarcadores de exposição em trabalhadores expostos a tintas.

11. **XX Congresso Pan-americano de Farmácia**, 2010. (Congresso)
.

12. **XXXVI Semana Acadêmica de Estudos Farmacêuticos**, 2010. (Outra)
.

13. **Curso Interação Medicamentos e Alimentos**, 2009. (Outra)
.

14. **Semana Acadêmica da Engenharia de Alimentos**, 2009. (Oficina)
.

15. **Simpósio sobre Terapias Inovadoras**, 2009. (Simpósio)
.

16. **XXI Salão de Iniciação Científica**, 2009. (Seminário)
.

17. **XXXV Semana Acadêmica de Estudos Farmacêuticos**, 2009. (Outra)
.

Organização de evento

1. GARCIA, S. C.; Limberger, Renata P; **WAECHTER, F.**; Nascimento, Sabrina; Brucker, Natália; Bulcão, Rachel; Freitas, Fernando; Moro, Angela M.
II Congresso Sul de Toxicologia Clínico-Laboratorial, 2011. (Congresso, Organização de evento)
Referências adicionais: Brasil/Português. Meio de divulgação: Vários

Totais de produção

Produção bibliográfica	
Artigos completos publicados em periódico	2
Trabalhos publicados em anais de eventos	2
Apresentações de trabalhos (Conferência ou palestra)	4
Apresentações de trabalhos (Congresso)	3
Apresentações de trabalhos (Simpósio)	1
Apresentações de trabalhos (Outra)	4

Produção técnica	
Curso de curta duração ministrado (outro)	1
Desenvolvimento de material didático ou instrucional	1

Eventos	
Participações em eventos (congresso)	6
Participações em eventos (seminário)	1
Participações em eventos (simpósio)	5
Participações em eventos (oficina)	3
Participações em eventos (encontro)	1
Participações em eventos (outra)	8
Organização de evento (congresso)	1