SPECIFIC CONTRACT

No. 03 (SC03)

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Endogenous formation of nitrosamines from drug substances

(GITox)

Deliverable 2 – Study plan

The study plan, to investigate the impact of gastrointestinal environment on the emergence of nitrosamines (NAs) from active pharmaceutical ingredients, lists all relevant parts and final conditions for evaluation. All planned steps were checked for plausibility and the reasons for these decisions were explained. In case changes are necessary during the experiments or e.g. changes in the composition of media, this is recorded. With regard to the results to be published at the end of the study, the evaluation of the experiments by study plan facilitates justification in the face of critical comments from outsiders. It should be noted that with regard to the final API selection, the literature review was used and results are presented again, as they were also relevant and representative for the study plan. However, these results were further refined and expanded to include decision criteria.

Content

1 Key personal involved in the project

Organisation	Key person	Role(s)
Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM)	Dr. Sylvia E. Escher	Database development, literature search, in silico QSAR calculations
Federal Institute for Drugs and Medical Devices (BfArM)	Dr. Matthias Vogel	Lead scientist LC-MS, incubations in artificial gastric juice
University of Bonn – Institute for Microbiology and Biotechnology (IMB)	Prof. Dr. Uwe Deppenmeier	Microbiomal and intestinal incubations

2 Aims and objectives

In the upcoming study it is of utmost importance to fill scientific gaps and highlight lacks of research data encompassing the impact of GIT conditions on the emergence of API-NA derivatives. Additionally, the here provided study plan was conducted to finally gather relevant data to present representative laboratory protocols and justificate adequate GIT models. Thereby, the following tasks were in the focus of the study plan:

- Presentation of method validation and key sample processing steps for accurate and sensitive detection of API-derived nitrosamines.
- Presentation of a representative model to study the nitrosation rate of drugs in artificial gastric juice.
- Use of physiologically evaluated nitrite concentrations in the stomach to simulate drug ingestion on an empty stomach as well as during food intake.
- Final selection and cultivation of most abundant microbiomal strains. Selection of model gut bacteria which are bioinformatically characterized by suitable nitrate and/or nitrite forming enzymes that might influence nitrosation reactions.
- Extension of the investigation models by possible suppression mechanisms by so-called nitrite scavengers (e.g. ascorbic acid in food) and their favourable influence on inhibition of nitrosation kinetics.
- Investigation of possible detoxification mechanisms or metabolic processes of API derived nitrosamines by microbial strains.

During the literature review and the final planning of the protocols, deviations from the originally planned experiments and study proposal arose, which will be explained in more detail here. Initially, it was planned to incubate drugs in simulated saliva or in the environment of the buccal cavity. However, since the residence time of most drugs in the mouth is very short and even fused or sublingual tablets rely on the drug being swallowed or absorbed quickly, this series of trials is no longer included in the final study plan. Also, high amounts of nitrate-rich foods, which may be converted into significant amounts of nitrite by oral bacteria, ultimately lead to a higher concentration of nitrite in the stomach, which is also reflected in the final physiological nitrite levels for the incubations in the artificial gastric

juice. Furthermore, the temperature in the artificial gastric juice should be kept physiological, i.e. at 37°C, in all experiments. A deviation did not seem logical, so that only the pH value and the nitrite concentration will play a role. The technical and biological replicates mentioned above are of utmost importance to corroborate the results. However, we will make the extent and nature of the replicates dependent on the initial experiments to what extent nitrosamines are generated at all. Since we want to perform as many experiments as possible across studies within a short period of time, the schedule for performing the technical and biological replicates will only be presented within the intermediate reports and discussed within the regular meetings with the EMA. Nevertheless, the initially planned numbers of technical replicates are 2, the numbers of biological replicates are 3 at different timepoints.

3 Final selection of test compounds

Taking into account the literature review and other relevant statistical data, 14 substances were finally selected to be included in the planned trials. This section partly contains repetitions and results from the literature review. However, these are of high importance here, once again for final explanation and justification of the selection, so that they are listed again. The final monographs of all substances and their nitroso derivatives are also included in the appendix. At least five criteria which mainly influenced the final API selection were considered and are as follows:

1. Significant structural deviations from low molecular nitrosamines like NDMA, NDEA, etc.
2. Presence of valid information and references concerning API-NA occurrence in the past and/or DNA alkylation accompanied by categorization as mutagenic/carcinogenic compound
3. Bulky residues within API without less information concerning nitrosation
4. pk_a considerations with regard to nitrosation tendency
5. Assure overlap with the selected compounds in two other EMA projects on NAs

All five parameters were considered equally and applied on the finally selected API.

Criterion 5 was evaluated under consideration of non-API nitrosamines with bulky residues which have been investigated via QSAR modelling. The term 'bulky' was introduced to draw a clear distinction from the previously well-documented low-molecular-weight nitrosamines. The focus is now on more expansive chemical groups such as extended ring systems / mesomerically stabilized aromatic systems, additional functional groups such as carboxylic acids or sulfonamides, but also halides. It can be assumed to have a potential steric hindrance on the various biological processes investigated in this project, namely metabolic activation, DNA adduct formation and repair processes. Representative APIs on the market with different, sterically demanding side chains were selected, such as aromatic groups, branched alkyl chains or hydroxylated side chains (please refer to figure 1). The main focus of the inclusion of pk_a values can be found in the range of aromatically and aliphatically substituted secondary amines. The former are found in the range of about pk_a = 4-6, the latter correspondingly in the range of 9-11. Many drugs which carry secondary amines can be found in these ranges, which is why they were included for closer examination on the basis of criterion 4. pk_a values for each API can be found in the respective monographs and section 7. Criterion 2 and 5 were taken into account by means of prior literature reviews in all projects. An excerpt was prepared as table 1.

Table 1: Summary of compound specific data for API-derived NAs; priority (P) 1 and 2 are given for testing in this project. Data on metabolic activation and experimental data on mutagenicity and carcinogenicity are cited from the NA masterlist compiled in the other two EMA projects. The data on metabolic activation is for most of the compounds predicted based on their structural properties.

CAS	Name	P*	General interest	Metabolic Activation	Mutagenicity / carcinogenicity
17608-59-2	N-Nitroso-ephedrine	2	Drug impurity, b(anta)agonist; data rich compound	Alpha-carbon hydroxylation on the methyl substituent is expected with significant DNA adducts formed	First Ames positive only in the presence of S9 mixture (+ S9)
29291-35-8	N-Nitroso-folic acid	2(1**)	Drug impurity; part of many nutritional additives, particularly for pregnant women; limited evidence on carcinogenicity	Predicted: alpha-carbon hydroxylation metabolism is limited by the presence of only one hydrogen on the alpha-carbons and presence of carboxylic acids resulting in high solubility	No data
55855-42-0	N-Nitroso-Nortriptyline	1	Drug impurity; antidepressant, EMA list	Alpha-carbon hydroxylation on the methyl substituent is expected; resulting large and bulky diazonium ion may result in cytotoxicity instead of DNA mutation.	No data
63779-86-2	N-Nitroso-hydrochlorothiazide	1	Diuretic compound for hypertension; ingredient	Electron-withdrawing effects, steric hindrance, solubility, considerations on the mechanism might affect the carcinogenic potential	Recent Ames positive
84418-35-9	N-Nitroso-propanolol	1	Drug impurity; □(anta)agonist	Potentially hydroxylation at two alpha positions, the isopropyl group and the substituted propyl function, formation of diazonium ion of the substituted propyl and	First evidence: Ames positive genotoxicity independent of the metabolic activation system (±S9)

				isopropyldiazonium ion.	
92432-50-3	N-Nitroso-trimetazidine	2	Drug impurity; anti-ischemic (antianginal) metabolic agent	Alpha-carbon hydroxylation followed by ring opening to form a diazonium aldehyde	No data
138768-62-4	N-Nitroso-metoprolol	1	Drug impurity; (anta)agonist	Predicted hydroxylation at two alpha positions, the isopropyl group and the substituted propyl function, formation of diazonium ion of the substituted propyl and isopropyldiazonium ion	Metabolic activation needed to get mutagenic
150494-06-7	N-Nitroso-fluoxetine	1	Drug impurity; antidepressant; drug no data	Alpha-hydroxylation predicted; large size of the potential adduct suggests a high level of cytotoxicity, frameshift mutations, and a lower level of mutations per adduct.	First evidence: Ames positive genotoxicity independent of the metabolic activation system (±S9)
1246819-22-6	N-Nitroso-desloratadine	1	Drug impurity; very common antihistamine	Alpha-hydroxylation leading to formation of a very large ether-ring potential adduct with a high level of cytotoxicity and a lower level of mutation per adduct	No data
2470278-90-9	N-Nitroso-rasagiline	2	Drug impurity; irreversible inhibitor of monoamine oxidase for Parkinsonism	Limited alpha-hydroxylation predicted. The large size of the potential adduct suggests a high level of cytotoxicity, frameshift mutations, and a lower level of mutation per adduct	No data

2490432-02-3	N-Nitroso-bumetanide	2	Drug impurity; anti-hypertensive (loop diuretic); no data	Moderate-level of alpha-hydroxylation and mutagenicity predicted due to steric hindrance	No data
2680527-91-5	N-Nitroso-duloxetine	2	Drug impurity; antidepressant; no data	Alpha-hydroxylation; the large size of the potential adduct suggests a high level of cytotoxicity, frameshift mutations.	No data
2708280-93-5	N-Nitroso-furosemide	1	Drug impurity; anti-hypertensive (loop diuretic); no data	Alpha hydroxylation; large size of the potential adduct suggests a high level of cytotoxicity, frameshift mutations, and a lower level of mutation per adduct	No data
2724616-80-0	N-Nitroso-lorcaserin	2	Drug impurity; weight loss drug	Alpha-carbon hydroxylation followed by ring opening to form a diazonium aldehyde	No data
2755871-02-2	N-Nitroso-varenicline	1	Drug impurity; API acts on nicotinic acetylcholine receptor; drug recalled, because of this impurity	Alpha-carbon hydroxylation followed by ring opening to form a diazonium aldehyde	No data
nocas-3	N-Nitroso-chloroquine	2	Drug impurity; malaria and rheumatoid arthritis drug	Poor or no alpha-carbon hydroxylation; only denitrosation is predicted	No data
nocas-5	N-Nitroso-enalapril	1	Drug impurity; anti-hypertensive, ACE inhibitors; no data	Alpha-carbon hydroxylation; limited metabolism and moderated mutagenicity is predicted. The large size of either potential adduct suggests a high level of cytotoxicity, frameshift mutations, and a lower level of	No data

				mutation per adduct.	
nocas-9	N-Nitroso-salbutamol	1	Drug impurity; (anta)agonist, recalled	Potential hydroxylation of alpha carbon, formation of tert-butyl-diazonium ion, considered of low reactivity to DNA.	First evidence: Ames positive only in the presence of S9 mixture (+ S9)
32635-81-7	N-Nitroso-betahistine	1	Drug impurity;	Structurally very similar to Nitroso-N-methyl-N-(2-phenyl)ethylamine; major route of this similar compound is alpha hydroxylation	No data on this compound, Nitroso-N-methyl-N-(2-phenyl)ethylamine is Carc_positive; no AMES
55557-03-4	N-Nitroso-methylphenidate	1	Drug impurity;	Alpha-hydroxylation leading to formation of a very large ether-ring potential adduct with a high level of cytotoxicity and a lower level of mutation per adduct	No data
85440-79-5	N-Nitroso-2methylindoline	2	Drug impurity;	Structurally similar to N-Nitrosoproline (7519-36-0); this compound has a minimal metabolism in rats; almost completely excreted unchanged in the urine; main route of activation via metabolism is decarboxylation to nitrosopyrrolidine, a powerful carcinogen	No data for N-Nitroso-2methylindolinbut for similar compound - N-Nitrosoproline, which is a true negative control for Ames test

*priority for testing in this project; ** if commercially available

Conclusion for final selection

So far, there have been no or only preliminary reports on mutagenicity or carcinogenicity of API related nitroso derivatives (see also Table 1). Therefore, this ranking criterion cannot be seen as decisive aspect for compound selection. As listed in precalculated QSAR data (see section 7), most of the pk_a values for secondary amines lay in the range between 8-10. If steric hindrance or other parameters do not have impacts on the nitrosation rate, most APIs have to be regarded equally. Exceptions are enalapril, furosemide, bumetanide, hydrochlorothiazide and folic acid. It seems, that these compounds

react more easily with nitrosating agents. Nevertheless, NAs with different structural properties were selected to ensure a high degree of overlap with the other two EMA projects, as structural features such as steric hindrance at the alpha position may have an influence on the metabolic activation and thus on the mutagenic potential of NAs. After evaluating the preselection of compounds, one of the most decisive criteria for compound reactivity seems to be adjacent groups next to the secondary amine incorporating inductive or mesomeric effects. Here, structural characteristics were highlighted and figured out in figure 1. Already reported API-NA contaminations in API bulk or market released drug formulations with seldom moieties or ring systems were chosen. At least, all selected compounds should be available as reference standards.

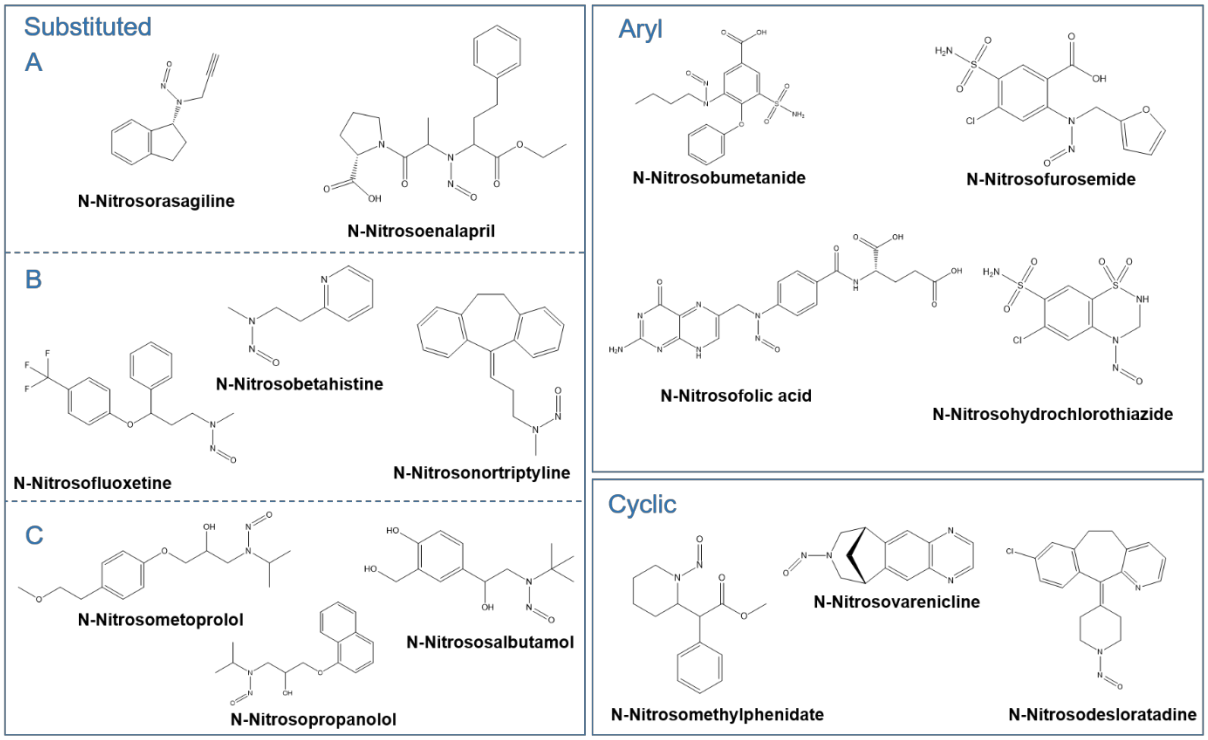


Figure 1: Overview on the structural properties of API-derived NAs (see NA-masterlist); API-derived NAs are subclassified according to the substituents next to the NNO group e.g. containing bulky alky side chains (substituted); adjacent to aromatic substructure (aryl); being part of a non aromatic ring system (cyclic). This project investigates the impact of different structural properties on the formation of Nitrosamines as well as their mutagenic/carcinogenic activity/potency. In the broad class “substituted” three subclasses can be identified. A) A broad class containing different types of alkyl substituents, B) NAs, which all contain a methyl side chain and different other chains which differ with regard to their sterical hindrance. C) NAs formed from the beta-(anta)agonists metroprolol, propanolol and salbutamol, they share a hydroxyl-group in ß-position to the NNO-group as well as an adjacent alkyl-group (propyl to tert-butyl) which differ in terms of sterical hindrance.

With the update of the document 'Questions and answers for marketing authorization holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products', limit values for further API nitroso derivatives have been added (EMA 2022). This suggests that these could also be critical and affected substances. It is interesting that now not only limit values for NDMA, NDEA, DIPNA and others are given, but also for nitrosovarenicline and nitrosorasagiline. Nitrosomethylphenidate and nitrosonorotriptyline are also newly listed. As the ongoing study has shown, there are only a few suppliers who offer derivatized drugs or manufacture them on request. Even the quantities of available substances declared as officially stocked proved to be partly unavailable upon request. However, some certified nitrosated

drugs could already be purchased in small quantities in advance, for preliminary trials. The advantage of the experimental approaches of the GITox study is the small amount needed for detection by LC-MS. Currently, we are in possession of all nitrosated drugs listed below except nitroso folic acid, nitrosobetahistine, nitrosofurosemide, nitrosovarenicline, and nitrosomethylphenidate. In the meantime, however, nitrosofolic acid, nitrosobetahistine, nitrosomethylphenidate, and nitrosovarenicline have already been ordered. Here, we are waiting for the official order confirmation. Since furosemide is widely used as a therapeutic agent and also, according to pk_a value, is well nitrosable, we would like to include the substance in the study. Unfortunately, this substance is not available or is associated with a very long delivery time. Therefore, nitrosobumetanide serves as a substitute substance, which is already available in house, but has a significantly lower market relevance as a drug.

Considering the mentioned facts, a final selection of priority 1 compounds is as follows. Thereby, the yellow marked compounds were declared as more susceptible for nitrosation towards the other compounds.

1. **Amitriptyline/Nortriptyline**
2. **Betahistine**
3. **Enalapril**
4. **Fluoxetine**
5. **Furosemide; alternative: Bumetanide**
6. **Hydrochlorothiazide**
7. **Loratadine**
8. **Methylphenidate**
9. **Metoprolol**
10. **Propranolol**
11. **Salbutamol**
12. **Varenicline**
13. **Folic acid**
14. **Rasagiline**

Subsequently, in order to further specify the protocols, it was determined which dose is adequate and representative to be included in the experimental protocol. We assumed the highest single doses of the respective substances that can be administered within a period of 6 hours. 6 h was chosen because within this time the tablet has already completely dissolved and its ingredients have passed through a large part of the gastrointestinal tract. Since some active ingredients are only available in a sustained release form, the lower release rate in the stomach must be taken into account accordingly. For all experiments concerning incubation in artificial gastric juice, it should be noted that these were carried out with commercially available preparations of the drugs (please see section 5.2). The single doses were chosen as follows (table 2).

Table 2: List of API amounts which are subjected to incubation

Name active product ingredient (API)	Highest daily single dose within 6h with regards to main indication (adults, 18-65 years, if possible:	Oral bio-availability	Source

	excluding retard tablets)		
Amitriptyline (Nortriptyline)	75 mg	53 %	National expert information
Betahistine	12 mg	102,8 % ≈ 100%	National expert information
Enalapril	20 mg	60 %	National expert information
Fluoxetine	60 mg	75 %	National expert information and Altamura *et al.* (Altamura, Moro et al. 1994)
Furosemide	80 mg	60-70 %	National expert information
Hydrochloro-thiazide	25 mg	70 %	National expert information
Loratadine	10 mg	40 %	National expert information and Madhav/Kishan (Madhav and Kishan 2017)
Methylphenidate	60 mg	90 %	National expert information and Kimko *et al.* (Kimko, Cross et al. 1999)
Metoprolol	100 mg (as metoprolol tartrate)	95 %	Claus-Jürgen Estler: Pharmakologie und Toxikologie. Schattauer Verlag 1999, ISBN 3794518950 (Estler and Schmidt 2012) National expert information
Propranolol	160 mg	90 %	National expert information
Rasagiline	1 mg	35 %	Lecht/Haroutinunian (Lecht *et al.* 2007)-

Salbutamol	8 mg (as retard tablet)	85 %	National expert information
Varenicline	1 mg	100 %	National expert information
Folic acid	550 µg	100 %	German Society for Nutrition 2022

4 Implementation and validation of analytical methods

4.1 Internal standards

The market situation and availability of stable isotope-labeled nitroso derivatives of drugs is very poor. However, it was possible to acquire d4-nitrosodeloratadine in advance of the studies, which will be used as an internal standard for quantification in the studies. Furthermore, it is planned to use stable isotope-labeled drugs of the compounds under investigation as surrogate molecules. From these compounds, non-quantitatively unknown amounts of nitroso derivative can be prepared by addition of tertiary butyl nitrite or classically by sodium nitrite at pH 1. The internal standard (ISTD) must always be added in constant quantities to the individual preparations. However, these volumes are independent of the actual concentration of the ISTD stock solution as long as the same solution and the same volumes per preparation are used. An overview of the preparation and availability is shown as an example in Figure 2 and table 3.

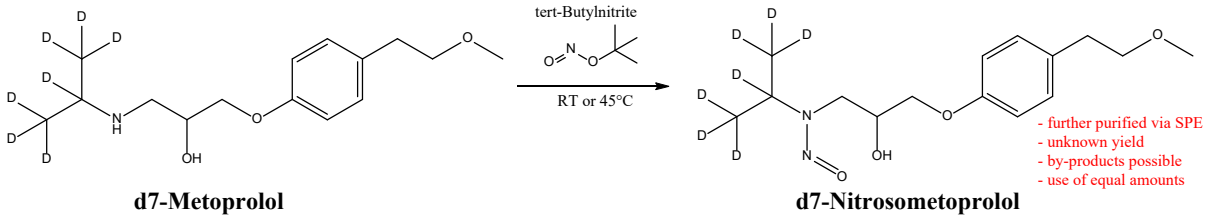


Figure 2: Principle of the synthesis of stable isotope-labeled ISTD in form of metoprolol. Excesses of tert-butylnitrite are further removed by evaporation (boiling point = 62°C) and subsequent solid phase extraction.

Table 3: Starting material for ISTD synthesis

CAS	ID	Provider	(synthesis or by order)
150494-06-7	Nitrosofluoxetine-d5	TRC	as Nitroso-fluoxetine-d5
no CAS	Nitrosonortriptyline-d3	TRC	synthesis from 203784-52-5/Nortriptyline-d3
no CAS	Nitrosobetahistine-d3	TRC	synthesis from 244094-70-0/Betahistine-d3
no CAS	Nitrosoenalapril-d5	biomol	synthesis from 349554-02-5/Enalapril-d5 (maleate)
no CAS	Nitrosofurosemide-d5	biomol	synthesis from 1189482-35-6/Furosemide-d5
no CAS	Nitrosohydrochlorothiazide-13C,d2	TRC	synthesis from 1190006-03-1/HCT-13C,d2
no listed CAS	Nitroso Desloratadine-d4	biomol	as N-Nitroso Desloratadine-d4
no CAS	Nitrosomethylphenidate-d10	TRC/LGC	synthesis from D-threo-Methylphenidate-d10 Hydrochloride
no CAS	Nitrosometoprolol-d7	biomol	synthesis from 1219798-61-4/Metoprolol-d7
no CAS	Nitrosopropranolol-d7	biomol	synthesis from 344298-99-3/Propranolol-d7
no CAS	Nitrososalbutamol-d9	biomol	synthesis from 1173021-73-2/Salbutamol-d9
no CAS	Nitrosovareniclin-d4	biomol	synthesis from 2183239-01-0/Vareniclin-d4
no CAS	Nitroso folic acid-d4	TRC	synthesis from 171777-72-3/Folic acid-d4

4.2 Work-up procedure and LC-MS measurement

After obtaining all nitrosated API-derived reference substances, they are analyzed in stock solutions of 1 µg/mL for substance-specific characteristics during mass spectrometric detection. First MS and MS/MS experiments are performed under electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) conditions. Regarding the ionization conditions, it can be said that smaller aliphatic nitrosamines can only be determined almost exclusively by APCI. However, since these are complex nitrosated compounds with several heteroatoms, it can be assumed that all measurements can be made by soft ionization, i.e. ESI. The first mass spectrometric experiment is an enhanced mass spectrum (EMS) experiment which detects ions in a predefined mass or m/z range. The linear ion trap in the third quadrupole (Q3) ensures an enrichment of the substances in the electric field and a jointly pulsed transmission of the analytical ions to the detector, which is reflected in a higher sensitivity. This is reflected in a higher sensitivity. Here, it is investigated whether, for example, a decay of the nitroso API compounds already takes place in the ion source and other output *m/z* values are to be expected. If this is the case, the declustering potential would have to be adjusted. The second experiment is a classical product ion spectrum, which however also uses the linear ion trap in Q3 and thus allows a higher sensitivity and resolution. By fitting the collision energy, corresponding MS/MS spectra are obtained for each compound, showing fingerprint signals characteristic of that compound. After the identification of corresponding quantifier and qualifier signatures, appropriate multiple reaction monitoring (MRM) experiments are set up, which allow a highly sensitive, selective and specific determination of the analytes. These MRM experiments are then validated and used for the entire study.

A 6500® triple quadrupol mass spectrometer from SCIEX is used throughout the study. The mass spectrometer is coupled to a Shimadzu Nexera® UPLC. For the first experiments, it is planned to use an Agilent Poroshell® 120 EC-C18, 100x3 mm, 2.7 µm particle size, as this has been very well established in routine work. This column also features the core shell technology, which is reflected in a strong improvement of all three parameters of the van Deemter equation, in particular due to improved uniformly packed solid phase particles, reduced void volume and fast mass transfer, resulting in very sharp or high chromatographic resolution signals. In the case that this column is not suitable for certain compounds, another equivalent column is used. It is planned to use ammonium format/formic acid buffer and acetonitrile as chromatographic eluent. The preparation of a gradient is obligatory and will finally be depend on the behavior of the individual analyte. Since a very large sample volume is expected, an analytical run should not take longer than 12min. This can only be guaranteed by a high flow rate.

All sample volumes mentioned in sections 4 - 6 are purified by solid phase extraction. In order to achieve an enrichment of both drugs and possibly nitrosated drugs, an HLB phase from Macherey-Nagel (Dueren, Germany) is used as solid phase, which allows both lipophilic and hydrophilic interactions with analytes. These are CHROMABOND® HLB columns, which are used with 1, 3, 5 or even 15 mL, depending on the sample volume. Purification is performed according to the manufacturer's instructions with successive equilibration, washing and elution steps with double distilled water and acetonitrile. After elution, volumes are appropriately evaporated by vacuum centrifuge for smaller volumes up to approximately 2 mL or nitrogen evaporator for volumes up to 15 mL. All samples are reconstituted with 100 µL of the chromatographic mobile phase composition at starting conditions and submitted to LC-MS analysis.

For the samples of the microbial experiments from section 6, a prior protein precipitation and cell lysis with acetonitrile is planned. For this purpose, acetonitrile (-20°C) is added aliquot to the cell culture in a 1:1 ratio after incubation. The suspension is then pretexted, centrifuged and the supernatant prepared for transport to the BfArM. Here, the internal standard is added and a large part of the acetonitrile is removed by nitrogen evaporator. The now predominantly aqueous solution is further purified by SPE as usual.

4.3 Validation

In order to detect the qualitative as well as quantitative conversion of active pharmaceutical ingredients (API) to nitrosamines, it is of utmost importance to perform this detection with valid methods. Not only the validity of the sample preparation but also the corresponding determination by instrumental analysis plays an important role. Prior to any in vitro incubations, which examine the pathway of the drug at critical sections of the gastrointestinal tract, the detection methods are validated for API-derived N-nitrosamines. International guidelines for the validation of bioanalytical assays are used, such as the ICH Q2(R2), EMA and EDQM/OMCL PA/PH/OMCL (13) 82 R5 guidelines (ICH 1995). It has to be emphasized, that the pure APIs in excess are not part of validation, as the expected amounts and resulting signals are out of the dynamic range as well all pure APIs cannot be seen as analytes of interest. The performance of the individual validation parameters is described in the next paragraph. The validation includes the work-up via solid phase extraction as well as the final determination method via LC-MS. Not part of the validation is the sampling procedure of the autosampler of the dissolution apparatus as well as the manual sampling during the microbial incubations. For the major of validation steps, a mixture of the corresponding drugs with placebo tablets in a 1:1 ratio serves as a validation matrix.

4.3.1 Specificity / Selectivity

For the determination of specificity and selectivity, 15 representative matrices in the form of substance-free placebo tablets, pure water for analytical purposes, ammonium format/formic acid/acetonitrile-water mixture and detectably nitrosamine-free tablets of all 14 drugs are examined. Single doses of these substances are used and processed as listed in Table 3. Subsequently, an amount of nitrosamine API reference is added to the same samples. Unambigiuous signals for specific ion transitions should appear compared to uncontaminated samples. Additionally, each reference API solution are fortified with its respective nitroso derivative to reveal any negative impact on the nitroso-API peak's intensity caused by the excess of pure API.

4.3.2 Linearity / Range

Since the conversion rate to nitrosamines is not yet known at the beginning, the linearity is first determined on the basis of the conversion rates of Gillat/Palmer and Ziebarth(Ziebarth and Teichmann 1980, Gillatt, Palmer et al. 1985). Here, drugs were converted to nitrosamine derivatives in the range of about 0.001 to 1 %. It is planned, initially as a starting value, to transfer this conversion rate to the single doses and thus to define the working range. For example, a dose of 50 mg incubated in 100 ml artificial gastric juice would result in a determination of linearity in the range 500 ng - 500 µg/100mL or 5 ng - 5 µg/mL. Within this range, 8 measuring points are to be determined. The data obtained during the linearity and accuracy studies will be used to assess the range of the method.

4.3.3 Limit of detection (LOD)

In accordance with the ICH Q2(R2) guideline, a consideration of the signal-to-noise ratio of the peak height of 3:1 is taken into account. At least 6 samples are measured that meet these conditions. The collected value is compared with the theoretically calculated detection limit, which results from the formula LOD= (3.3*σ)/s. Here σ is the standard deviation of the response and s is the slope of the linearity calibration curve. These values should give an approximately equal result.

4.3.4 Limit of quantification (LOQ)

In the same way as the determination of the LOD value, samples must meet at least a signal-to-noise ratio of 10:1 to be considered quantifiable. Again, 6 equal concentrations are used for each analyte that must meet this ratio. The collected value is compared with the theoretically calculated quantification limit, which results from the formula LOD= (10*σ)/s. Here σ is the standard deviation of the response and s is the slope of the linearity calibration curve. These values should give an approximately equal result.

4.3.5 Precision

Repeatability

To determine repeatability, four concentration levels are determined in sixfold repetition and the resulting standard deviation and relative standard deviation are calculated. The repeatability is determined at the LOQ value, and at a value corresponding to about 25, 50, and 75 % of the range. The imprecision may only be +/- 20 % at LOQ and +/- 15 % for the other three concentration levels.

Intermediate Precision

Taking repeatability into account, the intermediate precision is determined on three consecutive days and the relative standard deviations of the peak-to-area ratios between days 1 and 2, 1 and 3, and correspondingly 2 and 3 are calculated. Again, the deviations must not exceed +/- 20% at the LOQ or +/- 15 % for all other concentration levels.

In the event that the conversion to nitrosamines is higher or lower, the following precision limits, as shown in table 4, are used.

Table 4: Precision specification criteria

Component measured in sample	Precision
≥10.0%	≤ 2%
1.0 up to 10.0%	≤ 5%
0.1 up to 1.0%	≤ 10%
< 0.1%	≤ 20%

4.3.6 Accuracy

The accuracy is measured at 25, 50, and 75 % of the linearity maximum concentration and worked up in triplicate from placebo tablets previously fortified with nitroso API derivatives. After determining the mean value at each concentration range, the deviation is assessed by linear regression. Accuracy is defined as the ratio of the observed result to the expected result expressed as a percentage. Accuracy should be in the range of +/- 20%. In case of exceeding or even fall below the expected amounts, values will be reassessed with the following values (table 5).

Table 5: Accuracy specification criteria

Impurity content	Acceptable mean recovery
≥ 10%	98 –102%
≥ 1%	90 –110%
0.1 to 1%	80 – 120%
< 0.1%	75 – 125%

4.3.7 Robustness

To test the robustness of the method, certain critical factors of the determination method will be changed. It is planned to exchange the Poroshell EC-C18 column for another column with different dimensions and a different solid phase. The chromatography parameters and the gradient will remain the same. It will be investigated to what extent all substances can still be determined.

5 In vitro gastric incubations

5.1 Final composition artificial gastric juice

As discussed in the literature review, there are a variety of published artificial gastric juices ranging from simple standardized formulations to highly complex multi-component media. During our deliberations, and taking into account the literature, we concluded that an overly complex medium should not be used. In case a certain component accelerates or even inhibits the nitrification reaction,

the traceability of the causative agent might be difficult in multicomponent media and a direct attribution might not be possible. Nevertheless, physiological salts play an important role in maintaining the gastric environment, so phosphates, sulfates, and carbonates in particular should not be omitted. With the ulterior motive that the gastric juice in the filled or unfilled state reaches the next section of the GIT and can be further processed there by intestinal bacteria, we have come to the conclusion that the selected formulation of the gastric juice must not contain any components that are disadvantageous for the cultivation of the cultures to be examined. Thus, an effect of these species on the nitrosation of compounds or even detoxification mechanisms may not be observed. Following the SHIME medium for bacterial cultivation presented in section 6, a simplification is carried out which is representative for the unfilled as well as filled state of the stomach. The adaptation of this medium is a compromise between bacterial cultivation with essential nutrients and suitability as artificial gastric juice. The literature research clearly showed that the main cause for the formation of nitrosamines is the pH value and the amount of nitrite in the reaction environment. The inclusion of thiocyanate or ascorbic acid, for example, can of course stabilize and strengthen the reaction or suppress it accordingly. However, these components are only to be considered secondary, since they are not directly chemically converted, but only stabilize or neutralize the amount of reactant or nitrosyl cation. An influence on the main reaction by varying amounts of the components of the SHIME buffer are not to be expected. However, a reference sample with exclusively diluted hydrochloric acid at pH = pks nitrous acid = 3.15 is carried out to test this hypothesis.

The pH values will be adapted to respective pH values by titrating a 1M hydrogen chloride solution into the autoclaved modified SHIME medium. The current pH value will be controlled via a pH electrode. Final utilized volumes of 1M hydrogen chloride solution will be ascertained and can be used directly for the adjustment of buffers in the future. Respective nitrite concentrations in form of sodium nitrite will be added after autoclaving to evade nitrite degradation. Pepsin was added in reference to Foltz et al. with 50 µg/mL (Foltz, Azad et al. 2015). The final composition is listed in table 6.

Table 6: Modified SHIME reactor medium representing artificial gastric juice **prior** food intake. Modified after Molly et al., 1993 and Abbeele et al., 2010.

Compound	
Mucin	1 g
$NaHCO_3$	2.5 g
NaCl	2 g
K_2HPO_4	5.6 g
KH_2PO_4	4.4 g
Salt solution	1 mL
NH_4Cl	1 g
Pepsin (added after autoclaving)	50 mg
Dest. Water	Ad 1000 mL

Salt solution: 8 g $CaCl_2$; 8 g $MgSO_4$ x 7 H_2O ad 1000 ml

In reference to EUSES database the mean human daily intake of meat is 4.28 g / kg [body weigth] / d, which reflects a high meat consumption in the European Union. Referring to a person with 70 kg, the meat consumption lay at approximately 300 g meat / d. In reference to the German Society for Nutrition and the U.S. Department of Agriculture the mean protein concentration for meat is 20 g protein / 100 g meat. In combination with the intake of liquids during a meal (2 L/d equal to 500 mL four times a day, EUSES database), a total volume of 500 mL with 20 g peptone from meat for simulating in vitro gastric conditions after food intake is planned. Starch, xylan, mucine, and pectine

represent the content of biopolymers. Compared to the vitamin mix of the bacterial culture media, this one does not contain any folic acid, otherwise it is difficult to assess the results due to any other folic acid excess. The artificial gastric juice representing the filled status via food intake is listed below (table 7).

Table 7: Modified SHIME reactor medium representing gastric juice **after** food intake. Modified after Molly et al., 1993 and Abbeele et al., 2010

Compound	
Xylan from oat spelts	1 g
Glucose	1.8 g
Yeast extract	3 g
Soluble starch	3 g
Peptone from meat	20 g
Mucin	1 g
Pectin	1 g
$NaHCO_3$	2.5 g
NaCl	2 g
K_2HPO_4	5.6 g
KH_2PO_4	4.4 g
Salt solution	1 mL
Haemin solution (added after autoclaving)	1 mL
Vitamin solution (after Wolin et al., 1963*) + ascorbic acid 20 mg/mL (added after autoclaving)	1 mL
Vitamine K	0,1 %
NH_4Cl	1 g
Pepsin	25 mg
Dest. Water	Ad 500 mL

*20 mg biotin, 100 mg pyridoxine x HCl, 50 mg thiamine x HCl, 50 mg Na-Riboflavin, 50 mg nicotinic acid, 50 mg Ca-Pantothenate, 1 mg vitamine B12, 50 mg 4-Aminobenzoic acid, 50 mg α-Lipoic acid ad 1000 ml H_2O_{dest} (Wolin et al., 1963). Sterile filtered.

5.2 Incubations with varying nitrite and pH values at 37°C in time dependency

For all incubations pure APIs or respective salts will be investigated under light protection at respective concentrations. During the literature research, five pH values were identified which are of high interest for the incubation conditions to be chosen. These are pH 1.5, 3.15, 4, 6, and 7.4. The reason for this lies in the shift of the pH value in the stomach when different physiological conditions are present, as well as in the chemical reactivity of nitrite as the main component. Thus, pH 1.5 is the naturally occurring pH of the concentrated gastric fluid, corresponding to the stomach in an empty state. This is the situation when tablets are taken fasting. The selected pH value of 3.15 corresponds to the pk_a value of nitrous acid where the highest conversion rate or nitrosation of secondary amines with $pk_a > 5$ was observed (Mirvish 1975). pH 4 corresponds to the acidity after food intake and plays a major role especially for part 5.3. Here, pH is used to better assess the formation kinetics of nitrosamines. pH 6 was chosen to simulate the transition region to the duodenum. In general, the pH in the further section of the small intestine is between 6-7.4. The latter value is also found as the final and highest pH value used. Higher pH values in the alkaline range are excluded from the investigations, since no significant nitrosation rate is expected. With regard to the concentration of nitrite or the ratio of nitrate to nitrite to be used, it must be said that although there are some publications that demonstrate a certain molarity of nitrite in the stomach, the scatter within the publications is very large. The main problem

is still the instability and high decay rate of nitrite under strongly acidic conditions. Micromolar concentrations in the double-digit range are only measurable from a pH value of 4. This was demonstrated by Xu and Reed in a study in 1993. Often total amounts of nitrate/nitrate can only be co-measured, which is often done by enzyme immuno turnover. Following research literature, a concentration range was empirically defined to be investigated, which covers physiological amounts of nitrite well. This spans the range of 1 - 200 µM. Based on the fact that higher pH values stabilize nitrite concentrations at 10-60 µM at pH 3-8 (Watt, Sloan et al. 1984, Xu and Reed 1993, Gao, Karfunkle et al. 2021). The final nitrite concentration also strongly depends on nitrate intake (Suzuki, Iijima et al. 2003). Additionally, nitrite concentration increase with rising pH. The 95th percentile nitrite concentration was observed with less than 1 µmol/L at pH levels less than 4, less than 10 µmol/L at pH levels 4 to 5, less than 100 µmol/L at pH levels greater than 5 to 6.99, and less than 200 µmol/L at pH levels of 7 or greater (Gao, Karfunkle et al. 2021). Another rather unphysiological nitrite concentration of 1M serves as an extreme value to provoke the nitrosamine reaction. In the event that no significant drug conversion is observed at such a high concentration, this may indicate that the API is unresponsive and unsusceptible. As presented in table 3 each API, using the highest single daily dosage, will be probed. In contrast to affected gastric juice by fluid or food intake, the physiological resting volumes lie in the range of approximately 25-65 mL (25-50 mL (Hutchinson, Maltby et al. 1988), 35 mL (Mudie, Murray et al. 2014), 35-65 mL (Grimm, Koziolek et al. 2018)). Thus, total volumes of one dosage per 50 mL residual fluid will be probed at 37°C under light protection (amber glass). All APIs are checked for pre-existing nitrosamine contaminations. The incubation experiments are performed with a dissolution tester under normal aerobic atmosphere. As alternative, all experiments can be performed in light protected 96 well plates to reduce the consumption of material and API. At the timepoints 0, 5, 15, 30, 45, and 60 min aliquots of the reaction will be taken and suppressed with sulfamic acid to stop nitrite reaction. The respective internal standard (nitrosated stable isotope labeled API + stable isotope labeled API + by-products of chemical synthesis) is added directly after suppression of nitrite during sampling. Thus, losses during SPE purification can be compensated. Afterwards, samples are subjected to SPE purification and LC-MS measurement. After collecting all data per pH and nitrite range, it is planned to repeat the experiments as technical replicates, if the time limitation of the study allows it.

Table 8: Selected nitrite concentrations and pH values with corresponding justification written in italics. The shaded area is identical to the intended sampling in the upper left field.

pH / **Nitrite conc.**	pH 1.5 *pure gastric juice*	pH 3.15 *pH = pk_a HNO_2*	pH 4 *pH after food intake*	pH 6 *pH duodenum*	pH 7.4 *physiological pH*
1 µM *lower limit of physiological nitrite concentration*	sampling at 0, 5, 15, 30, 45, and 60 min				
33 µM *mean physiological nitrite concentration*					
200 µM *upper limit of physiological nitrite concentration*					
1 M *nonphysiologic; forcing nitrosation*					

It is expected from this experiment of the study that formation kinetics of nitrosamines from drug substances can be observed and calculated. Crucial points are whether certain structural features within the compound are sensitive to nitrosation at all, and if so, to what extent. This makes it possible to assess the susceptibility of other drugs to nitrosamine formation. In this part, the influence of nitrate should be excluded as far as possible, since the reduction of nitrate to nitrite is subject to enzymatic kinetics of buccal bacteria. This context plays more of a role in the ingestion of nitrate-rich foods and is examined in section 5.3.

While the incubation experiments with real dosages primarily contribute to drug safety and whether nitroso API formation in the stomach occurs at a certain dosage, the upcoming experiment deals with the structural susceptibility of substance classes to be nitrosated. Here, APIs are incubated in equimolar and increasing concentrations with an excess or equal amounts of nitrite ions for 2h. The extent to which nitrosamines are formed from API is determined according to endpoint determinations. For highly susceptible and reactive secondary amines, the relative yield would be expected to be similar for both high and low excess or equal amounts of nitrite. In the case of unreactivity, an increase in the amount of drug relative to nitrite would result in a slowing of the conversion and a worsening of the relative yield. All incubations should be performed at pH 1.7 corresponding to a 0.02 M HCl solution. The pH value was selected somewhat lower, since the solution is not buffered and a consumption of protons and thus an increase in the pH value can be assumed. An overview of the approach is given in form of figure 3.

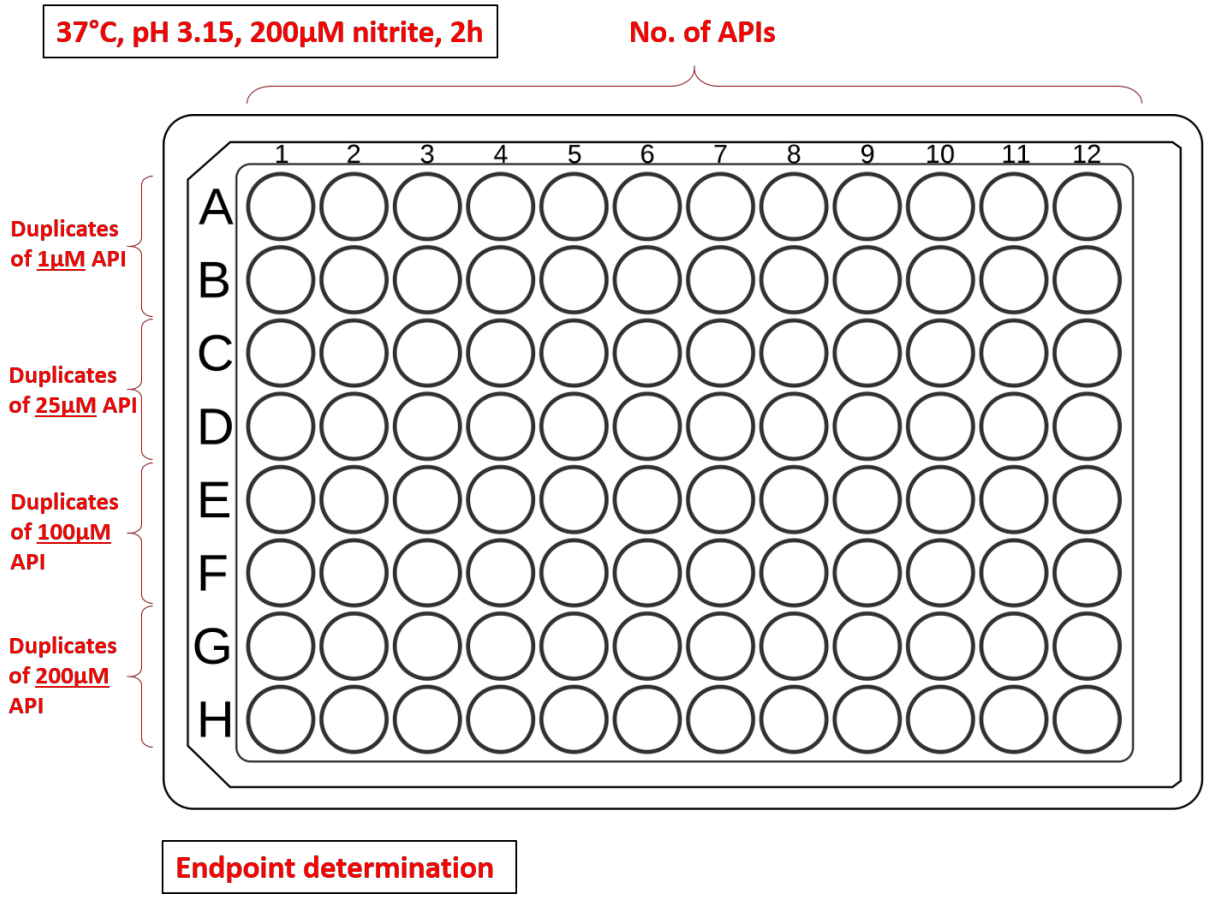


Figure 3: Planned experiment to assess the structural differences with regards to nitrosation at equal concentrations.

5.3 Impact of food/nutrients on API nitrosation

In a similar approach compared to point 5.2, the influence of food and food components/nutrients on the nitrosability of drugs is investigated. An essential difference compared with the kinetics calculation from point 5.2 is the supply of nitrate via food and the enzymatic conversion to nitrite by oral cavity bacteria. The increase in the amount of nitrite in the stomach can be the result. Another point is the higher volume of gastric juice due to possible liquid intake during eating or generally due to the volume of the food. A very important role will also be played by peptides or amino acids like proline, which, due to their amine functions, are able to intercept nitrosyl cations and thus compete with the secondary amines of the drugs. The addition or uptake of nitrite scavenging molecules such as ascorbic acid, which is also present in high quantities in food, will also play an important role in this experiment. The reaction equation of nitrite inactivation is given below.

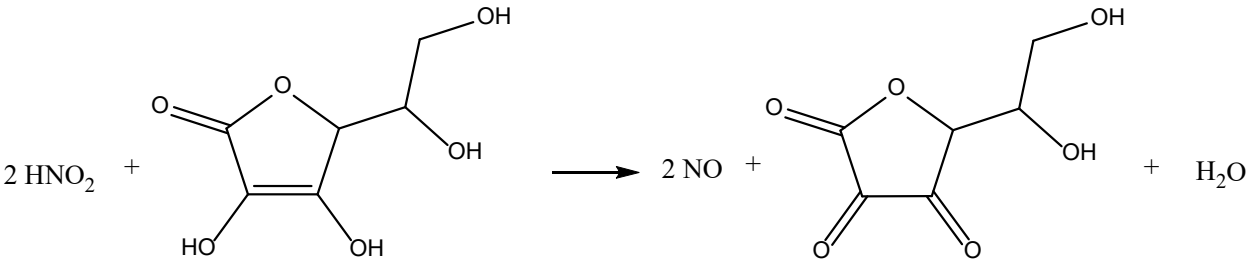


Figure 4: Nitrite deactivation via ascorbic acid

It should be noted, however, that a variety of possible reactions can influence nitrosation in this experiment. Which parameter ultimately leads to an increase or decrease in nitrosation must be discussed. However, a non-influence of the nitrosation rate compared to the fasting gastric medium is also quite conceivable.

It can be discussed to what extent the nitrite concentration in the stomach increases with high nitrate intake and further conversion by oral cavity bacteria or generally through the consumption of nitrite-containing foods. In both cases, the final nitrite concentrations are individually strongly dependent on the type of food consumed, personal eating habits, and of course the conversion rate by bacteria. It can therefore be concluded that these values can vary greatly and that a representative true value does not exist. The idea is, in order to investigate the effect and influence of food, to adopt the same physiological nitrite concentrations from 5.2. Then, the conversion rates of nitrosation in the fasting artificial gastric environment are compared with those in the presence of nutrients.

A separate experiment will be carried out in accordance with point 5.2 in order to consider possible recommendations and requirements for future drug preparations. In the case that drugs with $pk_a > 5$ are subject to a strong and at pH 3.15 maximum nitrosation in the stomach, the question arises to what extent this reaction can be prevented. Since an incubation at pH 3.15 without vitamin additives is already carried out under point 5.2, a further incubation at pH 3.15 with vitamin additives logically follows as a compromise between nitrite stability and maximum reaction rate. This incubation is carried out without artificial food components, but is to be seen as part of point 5.3, since a vitamin supplement is added.

Table 9: Planned series of experiments of incubations in the presence of food components and the addition or omission of vitamins, especially ascorbic acid.

pH / **Nitrite conc.**	pH 4 *pH after food intake* ***(- vitamin cocktail)***	pH 4 *pH after food intake* ***(+ vitamin cocktail)***	pH 3.15 *pH = pk_a HNO_2* ***(+ vitamin cocktail)***
1 µM lower limit of physiological nitrite concentration	sampling at 0, 5, 15, 30, 45, and 60 min		
33 µM *mean physiological nitrite concentration*			
200 µM *upper limit of physiological nitrite concentration*			
1 M *nonphysiologic; forcing nitrosation*			

6 In vitro intestinal incubations

Data on degradation and toxification of APIs to API-NAs cannot be generated in *in vivo* studies. Therefore, *in vitro* assays will be utilized to mimic gastrointestinal (GIT) conditions. The impact of aerobic and anaerobic microbiomal strains in the GIT, to trigger API-NA formation, is part of the study. As indicated in the research proposal (objective 3), cultivating experiments with representative microbiomal strains like *Helicobacter pylori*, *E. coli* and artificial intestinal flora will be performed. Selected API (Tab. 3) will be added to the cultures to predict the impact of the microbiome on the arise of API-NA. The products will be analyzed by liquid chromatography and will be identified by tandem mass spectrometry.

In principle, the influence of intestinal bacteria on API-NA formation can happen in form of two scenarios: 1) Bacteria with nitrate reductases can form NO_2^- as a reactive agent to produce API-NA. However, chemical nitrosation only occurs in significant amounts when acidic pH values are present, which tends not to be the case for the small and large intestine (pH 6-8). 2) It is a possibility that API are taken up by bacterial cells and are then enzymatically converted to API-NA in the cell without the influence of the pH value. The bacterially mediated reaction is probably catalyzed by bacterial enzyme systems and proceeds much more rapidly at neutral pH than the chemical reaction. Such reactions were observed in *E. coli* and other enterobacteria, as well as in *Pseudomonas* strains and lactic acid bacteria (Calmels et al. 1987; 1991; Ralt et al. 1988; Suzuki and Mitsuoka 1984). The nature of the enzyme responsible for nitrosation is not yet clear (Zumft, 1993). In summary, it is evident that the knowledge about bacteria N-nitroso compound (NOC) synthesis in the human gut is very limited. While lactobacilli are present in abundance in the small intestine, the other mentioned bacterial organisms are found very rarely in the gut. The most prominent bacteria of the human colon (*Bacteroides* species and relatives of the order Bacteriodales or members of the phylum Firmicutes) were not yet tested for their ability to form nitrosamines in the gut. This will be the main task in this part of the project.

6.1 General approach

All bacteria (except *H. pylori* see 6.5.a) will be cultivated anaerobically in an appropriate medium supplemented with selected API. To achieve a high throughput, cultivation is performed in a 48-well plate with a cultivation volume of 500 µl. Incubation occurs at 37 °C in an Infinite 200 PRO NanoQuant Microplate Reader (Tecan, Männedorf, Switzerland), which allows the optical density to be recorded every 20 min to monitor cell growth. For cultivation under anoxic conditions, the plate reader setup will be introduced into an anoxic chamber and maintained in a CO_2 / N_2 / H_2 (20 % / 79 % / 1 %) atmosphere. Precultures of all species will be grown separately to an optical density of about 1.0. The individual species are then mixed and used to inoculate the wells of the plate. First, the cultivation will be carried out for 24 h in order to investigate whether a formation of API-NA takes place. Based on this, kinetic studies will then be performed, meaning every hour a sample will be analyzed of potential API-NA formation. Acetonitrile will be added to the samples in equal volume (500 µl) to stop enzyme reaction and to lyse the cells. The samples will be stored at -80 °C and transferred to BfArM for analysis.

6.2 Selected microorganisms

a) *Escherichia coli*: It is the most important model organism in life science research. The cells are Gram-negative, facultative anaerobic, and commonly found in the lower intestine of humans in relatively low numbers. Most *E. coli* strains are harmless, but some serotypes (EPEC, ETEC etc.) can cause serious

food poisoning. Virulent strains can cause gastroenteritis, urinary tract infections. The harmless strains are part of the normal microbiota of the gut. We will work with *E. coli* MC4100 because this strain is not virulent and grows well under anaerobic conditions.

b) *Helicobacter pylori*: It is a gram-negative, helical bacterium usually found in the stomach. *H. pylori* infection usually has no symptoms but sometimes causes gastritis (stomach inflammation) or ulcers of the stomach and is involved in cancer development. The strain requires oxygen, but at lower concentration than in the atmosphere. *H. pylori* usually burrows into the mucus and avoids stomach lumen with acidic pH. The organism is able to neutralize the acid in its environment by producing large amounts of urease which forms NH_3 and CO_2 from urea.

c) *Veillionella atypica* and d) *Limosilactobacillus reuteri* (former *Lactobacterium reuterii*) colonize the small intestine and will be used to analyze whether microbial API-NA formation occurs in the upper GIT (additional experiments, not indicated in the grant proposal).

e) Anaerobic gut bacteria:

The aim of this part of the project is to cultivate representative microbiomal strains present in the intestinal flora and to add APIs to predict the impact of the microbiome on the arise of API-NA. The major questions whether these gut bacteria are capable to trigger endogenous nitrosamine formation from APIs. The great challenge of this approach is the fact that the microbiota in the colon is highly diverse and differs from human to human. More than 1500 species were detected indicating that the number is too high to analyze all these bacteria separately. Hence, the major task is to mimic an average human microbiome. Several publications are available that describe numerical analysis of the microbiota in the colon of healthy persons (e.g. The Human Microbiome Project Consortium, 2012; King et al. 2019; Forster et al. 2019). Members of the Firmicutes and Bacteroidetes phyla make up a majority of the bacterial species that are present in the human colon. With respect to a core species concept, 84 organisms are present in the colon of almost all humans (King et al. 2019). We selected 10 of the 84 core species, which are present in high abundance in the human colon representing members of the most prominent phyla Bacteroidetes, Firmicutes and Proteobacteria. The composition of artificial gut microbiota, which will be used in our experiments is shown in Tab 10. Based on the findings of King et al. 2019 on average 55% of the core microbiota is covered by our species collection. This selection of organisms covers also the most abundant genera found in the human gut (*Bacteroides, Phocaeicola, Eubacterium, Faecalibacterium, Ruminicoccus, Escherichia, Roseburia and Bifidobacterium*). Hence, the species function as model organism of the respective genus. These genera cove ~ 83 % of all gut bacteria.

Table 10: Selection of gut bacteria

Organism	Maximal proportion (%)[a)]	Average abundance (%)[b,c)]
Phocaeicola dorei	42.5	17.4
Phocaeicola vulgatus	30.8	15.0
Agathobacter rectalis[d)]	42.1	6.2
Bacteroides xylanisolvens	25.6	4.9
Faecalibacterium prausnitzii	14.3	3.5
Hominimerdicola aceti[e)]	60.3	2.5
Escherichia coli	98.1	1.9
Alistipes shahii	8.2	1.8

Roseburia intestinalis	4.8	1.1
Bifidobacterium longum	10.3	0.7

a) Individuals possessing the highest number of respective bacterium
b) Average abundance from 127 healthy humans
c) On average 55% of the core microbiota is covered by the species collection
d) former *Eubacterium rectale*
e) former *Ruminococcus bicirculans*

f) Bacteria of the intestinal tract possessing nitrate or nitrite reductases (additional experiments, not indicated in the grant proposal)

Bacteria commonly metabolize nitrogen-containing compounds to enable incorporation into biomass or for respiration to conserve energy. Most of the organisms in the intestinal tract will probably use NH_4^+ or amino acids as nitrogen source. However, we performed a detailed bioinformatic analysis to identify genes encoding nitrate or nitrate reductases that could be involved in the nitrogen metabolism.

Broadly, nitrate reduction is classified into different major pathways (Koch et al. 2017; Sparacino-Watkins et al. 2014; Kox and Jetten 2015; Brittain et al. 1992; Maia and Moura 2014). Respiratory NO_3^- reduction, catalyzed by membrane-bound nitrate reductase (Nar), or periplasmic nitrate reductase (Nap), is the major electrochemical respiratory pathway for bacterial energy conservation under hypoxia via generation of a proton motive force, serving as a two-electron sink in the final step of ATP generation via the respiratory electron transport chain (Welte and Deppenmeier 2014). In addition, many bacteria contain an assimilatory nitrate reductase (Nas) in the cytoplasm that, in combination with a soluble nitrite reductase, produces NH_4^+, which is used as nitrogen source for cell mass production. Nitrite may be subjected to further reduction by the action of respiratory nitrite reductases. Here we can distinguish between NH_4^+-forming nitrite reductases (Nrf and Nir systems) and respiratory NO_2^- reductases that produce NO.

Our bioinformatic analysis of 110 genomes of species belonging to the most important genera of the human gut indicated that genes encoding NO_3^- or NO_2^- reductases or not widespread among these microorganisms, indicating that NH_4^+ or N-containing organic compounds are used for N-supply. However, a few organisms, which are present in relative high abundance in the gut, showed a variety of genes encoding different types of NO_3^- or NO_2^- reductases (Tab. 11). The enzymes could have two effects: 1) The conversion of NO_3^- to nitrite by NarG, NapA, and NasA could contribute to an increased nitrite concentration supporting the formation of API-NA. 2) The reduction of NO_2^- to NH_4^+ by NasB, NrfA and NirB could reduce the nitrite concentration and could contribute to detoxification, thereby preventing API-NA formation. We plan to include the bacteria shown in Tab. 10 in our analysis to verify the effect of NO_3^- or NO_2^- reductases on API-NA formation (section 6.2.e). We will also analyze the change in nitrite concentrations. For this purpose from each well a 50 µl sample will be taken for the analysis of the nitrite concentration. If the nitrite content decreases during the experiment, it can be assumed that nitrite reductases reduce NO_2^- to NH_4^+. If the nitrite concentration increases, this could indicate a reduction of NO_3^- by nitrate reductases. Correlations with the formation of API-NA may follow.

Table 11: Abundant gut bacteria with genes encoding NO_3^- or NO_2^- reductases

Organism Name	Isolation Body Site	NasA	NarG	NapA	NasB	NrfA	NirK
Bacteroides xylanisolvens	gastrointestinal_tract					2938062062	
Escherichia coli K-12	gastrointestinal_tract	646314162	646313148	646314162	646315332	646316056	
Lactobacillus reuteri	gastrointestinal_tract		2674094276				

The table shows the scientific name of the bacteria and the identification numbers of genes (IMG database) encoding potential NO_3^- reductases (NarG, NasA, NapA) and NO_2^- reductases (NasB, NrfA, NirK), respectively.

6.3 Nitrate and nitrite concentrations

The next question is the concentration of nitrate and nitrite that reflect the conditions in the stomach. Nitrate in the stomach has three sources: (1) ingested dietary nitrate, (2) enterosalivary circulation of nitrate, and (3) nitrate secretion from circulation (Packer and Leach 1991). Food contributes about 80 % in the exogenous nitrate intake (Archer 2002). Nitrate levels in vegetable and animal food vary widely (1-10,000 mg kg^{-1}) (Joint FAO/WHO Expert Committee on Food Additives, 1995), and therefore, nitrate intake varies considerably between subjects. According to a systematic review, median nitrate intake in 51 studies that included healthy subjects was ~1700 µmol day^{-1}) (Babateen et al. 2018; Ghasemi 2022). In accordance, total dietary exposure to nitrate ranges from ~900-3500 µmol day^{-1} as estimated by the International Agency for Research on Cancer (2010). As mentioned above about 20% of the nitrate in the stomach derives from enterosalivary circulation and nitrate secretion from circulation. Hence, the total nitrate supply is in the range of 2000 µmol/d. About 10 % of the total NO_3^- amount is reduced to NO_2^- by bacteria in the saliva. Assuming three meals per day and a stomach volume of 2 L the concentration of nitrate is then in the range of 300 µM directly after meal. According to Packer and Leach (1991) normal human intragastric nitrate levels are around 108-520 µM. Kondo et al. (2000) also published representative concentrations of nitrate in the gastric juice at a mean pH of 2.6 with 130 µM for nitrate. These results indicate that our calculation of the nitrate concentration in the stomach is in the right range. In summary, we will use a nitrate concentration of 300 µM in our experiments with gastric bacteria (*Helicobacter pylori*) and with *Veillionella atypica* and *Limosilactobacillus reuteri* which colonize the small intestine (section 6.2). A couple of publications are also available to calculate the NO_3^- concentration in the colon. These values range from 0.2 to 257 µmol/L (Rowland et al. 1991, Kälbe et al. 1990, Saul et al. 1981). We will use a concentration of 65 µM nitrate in our experiments with colon bacteria (section 6.2) which is the average of the value indicated in the literature.

Amounts of nitrite can be provided physiologically by the degradation of the signalling molecule nitric oxide or even provoked by bacteria in the oral cavity via nitrate reduction (Lundberg et al. 2008; Jensen 2009; Ma et al. 2018). Another nitrite source is diet, in particular by consuming sodium or potassium nitrite conserved meat products. It was calculated that about 93 % of the total ingested nitrite is derived from nitrate reduction in the saliva, and only 7 % comes directly from food (Archer, 2002). Because 10 % of the total $NO_3^-$is reduced to NO_2^- by bacteria in the saliva (see above) there is an intake of about 200 µmol/d (taking into account 2000 µmol NO_3^-/d). Assuming three meals per day and a stomach volume of 2 L the concentration of nitrite is then in the range of 33 µM directly after meal. From our literature search it became evident that because the instability under acidic pH values (pH 1-4), direct measurements of nitrite in the gastric stage are hard to perform. At higher pH values, ranging from pH 5 to 8, nitrite was determined at approximately 50-62 µM in the stomach (Watt et a. 1984). Kondo et al. (2000) determined a nitrite in the gastric juice at a pH of 2.6 with 14 µM . Xu and Reed in 1993 found nitrite concentrations of 0-650 µM depending on the pH value in the stomach (54 µM at pH 7). In summary, our theoretical calculation of 33 µM nitrite in the stomach match the values found in the literature. Therefore, we will use 33 µM nitrite in our experiments with *H. pylori* (stomach), *Veillionella atypica* and *Limosilactobacillus reuteri* (small intestine). For the large intestine values of 0.4

to 148 µM nitrite were reported (Hughes et al. 2001, Cross et al. 2003, Saul et al. 1981, Kälbe et al. 1990). The average of these values is about 20 µM. Hence a concentration of 20 µM nitrite will be used in the experiments with colon bacteria.

6.4 Applied API concentration

In order to investigate whether bacteria of the GIT are capable of forming API-NA, the maximum daily dose of the respective API is used in the experiments conducted. Therefore, it can be examined under maximum conditions whether API-NA formation takes place at all. If microbial API-NA formation is then detected, kinetics can be performed with lower concentrations of the API and also varying the nitrate and nitrite concentrations. Assuming that the stomach volume is about 0.5 liters after food intake and that content is ultimately passed to the small intestine, the concentrations of the APIs to be testes are calculated accordingly on their highest single dose (administered within a period of 6 h) per liter stomach volume (s. table 12). Some of the APIs are solved in DMSO, so as a control, the ability to form API-NA of the organisms will be studied with the appropriate concentration of DMSO.

Table 12: List of API and their concentrations used for the cultivation experiments[1)].

Name (API)	Concentration (mg/L) in the assay	Concentration in mM	Stock concentration
Amitriptyline (Nortriptyline)	150	0.48	48 mM in DMSO
Betahistine	24	0.072	7.2 mM in H_2O
Enalapril	40	0.08	8 mM in DMSO
Fluoxetine	120	0.344	34.4 mM in H_2O
Furosemide	160	0.48	48 mM in DMSO
Hydrochlorothiazide	50	0.168	16.8 mM in DMSO
Loratadine	20	0.052	5.2 mM in DMSO
Methylphenidate	120	0.44	44 mM in H_2O
Metoprolol	200	0.6	60 mM in DMSO
Propranolol	320	1.08	108 mM in H_2O
Rasagiline	2	0,0076	0,76 mM in H_2O
Salbutamol	16	0.068	6.8 mM in H_2O
Varenicline	2	0.0096	0.96 mM in H_2O
Folic acid	10	0.023	2.3 mM in H_2O

According to table 3 taking into account a stomach volume of 0.5 L.

6.5 Medium composition

a) Stomach (*H. pylori*)

H. pylori DSM 21031: Defined stomach juice is probably not suitable for growth experiments since *H. pylori* proliferates mainly in the mucus layer of the stomach with different substrate supply that is not exactly known and difficult to reproduce. Hence, we will use a published complex medium for growth of *H. pylori*. The organism will be grown in Brucella medium supplemented with 5 % fetal bovine serum (Gibco, sterile, heat inactivated) under a microaerobic atmosphere (Thermo Scientific™ Oxoid™ CampyGen; 85% N_2, 10% CO_2, 5% O_2) at 37°C with shaking at 100 rpm in a BD GasPak™ EZ container. If required, the organism is also cultivated on TSA agar plates supplemented with 5 % horse blood under the above mentioned microaerobic conditions. To test for the formation of API-NA, 300 µM nitrate or 33 µM nitrite is added to the Brucella medium (see 6.3). *H. pylori* is cultured in a volume of 20 ml Brucella medium with the appropriate API. A culture of *H. pylori* without the respective API serves as a control. First, sample analysis is performed after 24 & 48 h (stationary phase of *H. pylori* is reached only after more than 48 h (Olfat et al., 2002)) to first investigate possible formation of API-NA.

Subsequently, if API-NA formation is detected after 24 & 48 h, kinetics will be established. Whereas hourly samples will then be taken. Sampling is done by taking 1 ml of culture and adding equal volume of acetonitrile. The samples will be stored at -80 °C and transferred to BfArM for analysis. The respective composition of media are listed below (table 13 and 14).

Table 13: Composition of Brucella Medium for Helicobacter pylori cultivation.

Brucella Medium	
Casein Peptone	15 g
Glucose	1 g
Meat Peptone	5 g
Sodium Bisulfite	0.1 g
NaCl	5 g
Yeast Extract	2 g
Fetal bovine serum (sterile)	5% (added after autoclaving)
H_2O_{dest}	1000 ml
pH	7.0 ± 0.2

Table 14: Composition of TSA Agar

TSA Agar	
Casein Peptone	15 g
Soybean Peptone	5 g
NaCl	5 g
Agar	15 g
Horse blood (sterile, Defibrinated)	5 % (added after autoclaving)
H_2O_{dest}	1000 ml

b) Small Intestine (*Veillionella atypica* and *Lactobacterium reuteri*)

The small intestine or small bowel is an organ in the gastrointestinal tract where most of the absorption of nutrients from food takes place. It is therefore, extremely difficult to prepare a medium that reflects the nutrient availability of the entire small intestine. The content of the stomach is gradually released into the duodenum, so that a high concentration of nutrients can be expected in this area. Therefore, a nutrient-rich medium consisting of proteins, amino acids, carbohydrates, oligosaccharides, monosaccharides, fats and dietary fiber is used to grow the bacteria of the small intestine. For this purpose, we will use the published SHIME® (Simulator of Human Intestinal Microbial Ecosystem) medium. The SHIME® model is currently the most representative *in vitro* technology that mimics the entire gastrointestinal tract including the stomach, the small intestine and the colon (Van de Wiele et al. 2015). The basic concept of SHIME® was developed by Molly et al. in 1993. Here, the basal medium of the SHIME® reactor, modified after Van den Abbeele et al. 2010, is used for cultivation of bacteria of the gastrointestinal tract (see table 15). As test organisms for the small intestine, *Veillonella atypica* DSM20739, and *Lactobacillus reuteri* DSM 20016 will be used. The medium is similar to the artificial gastric juice after food intake (section 5.3). Here, cysteine is added which removes O_2 to allow an anaerobic growth of the bacteria. To test for the formation of API-NA, the respective API (see 6.4) and 300 µM nitrate or 33 µM nitrite are added to the modified SHIME medium (see 6.3). After 24 h acetonitrile will be added and samples will be stored at -80 °C. For further investigations, kinetic studies will be performed by analyzing API-NA formation hourly (additional experiments, not indicated in the grant proposal).

Table 15: Modified SHIME® reactor medium for simulation of the small intestine. Modified after Molly et al. (1993) and Van den Abbeele et al. (2010).

Compound	**Per liter**
Xylan from oat spelts	1 g
Glucose (separately autoclaved)	1.8 g
Yeast extract	3 g
Soluble starch[a]	3 g
Peptone from meat	3 g
Mucin[b]	1 g
Pectin[c]	1 g
L-cysteine (separately autoclaved)	0.5 g
$NaHCO_3$[d]	2.5 g
NaCl[d]	2 g
K_2HPO_4[d]	5.6 g
KH_2PO_4[d]	4.4 g
Salt solution[e]	1 ml
Vitamin solution (after Wolin et al., 1963) [f]	1 ml
NH_4Cl[g]	1 g
Vitamin K1 solution [h] (sterile filtered)	0.1 % (v/v)
Bile salts[i]	0.5 g
Hemin solution[j] (sterile filtered)	5 mg
Resazurin	1 mg
H_2O_{dest}	Ad 1000 ml

a: oxidized corn starch.
b: 75-95 % Porcine gastric mucosa.
c: ≥ 20 % pectin from apples.
d: Salt concentrations were calculated from human fecal data; 130 – 150 mM Na^+ and K^+, 30 mM HCO_3^-(Rao, 2011).
e: 8 g $CaCl_2$; 8 g $MgSO_4$ x 7 H_2O ad 1000 ml H_2O_{dest}
f: 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine x HCl, 50 mg thiamine x HCl, 50 mg Na-Riboflavin, 50 mg nicotinic acid, 50 mg Ca-Pantothenate, 1 mg vitamine B12, 50 mg 4-Aminobenzoic acid, 50 mg α-Lipoic acid ad 1000 ml H_2O_{dest} (Wolin et al., 1963). Sterile filtered.
g: ammonia concentration was calculated from human fecal data ~20 mM NH_4^+ (Wilson, 1968; Wrong & Vince, 1984; Eklou-Lawson et al., 2009, Wang et al., 2012).
h: 0.1 ml Vitamin K1 mixed with 20 ml 95 % ethanol
i: 50 % cholic acid sodium and 50 % deoxycholic acid sodium salt (Li et al., 2019).
j: 50 mg hemin mixed in 1 ml 1 M NaOH ad 100 ml H_2O_{dest}.

c) Large Intestine (Microorganisms in Tab. 1 and 2)

In the large intestine, water is mainly removed from the food pulp, thereby thickening it. Since almost all nutrients are absorbed in the small intestine, a slimmed-down version of the modified SHIME® reactor medium is used to simulate the reduction of nutrients. Salts, Cysteine, Hemin and especially polymers resistant to human digestion were excluded from this reduction because dietary fiber (xylan, pectin) are not hydrolyzed until they reach the large intestine (de Vos et al., 2022). Mucin is added because the mucus layer of the colon is a versatile substrate for many gut bacteria. As test organisms for the large intestine, *Phocaeicola dorei DSM 17855, Phocaeicola vulgatus DSM 1447, Agathobacter rectalis DSM 17629 (former Eubacterium rectale), Bacteroides xylanisolvens DSM 18836, Faecalibacterium prausnitzii DSM 17677, Hominimerdicola aceti* DSM 102216 (former *Ruminococcus bicirculans*), *Alistipes shahii* DSM 19121, *Roseburia intestinalis DSM 14610* and *Bifidobacterium longum subsp. infantis* DSM 20088 will be used.

Table 16: Modified SHIME® reactor medium for simulation of the large intestine. Modified after Molly et al. 1993 and Van den Abbeele et al. 2010.

Compound	**Per liter**
Xylan from oat spelts	1 g
Glucose (separately autoclaved)	1.8 g
Yeast extract	0.3 g
Soluble starch[a]	0.3 g
Peptone from meat	0.3 g
Mucin[b]	1 g
Pectin[c]	1 g
L-cysteine (separately autoclaved)	0.5 g
$NaHCO_3$[d]	2.5 g
NaCl[d]	2 g
K_2HPO_4[d]	5.6 g
KH_2PO_4[d]	4.4 g
Salt solution[e]	1 ml
Vitamin solution (after Wolin et al., 1963) [f]	1 ml
NH_4Cl[g]	1 g
Vitamin K1 solution [h] (sterile filtered)	0.1 % (v/v)
Bile salts[i]	0.5 g
Hemin solution[j] (sterile filtered)	5 mg
Resazurin	1 mg
H_2O_{dest}	Ad 1000 ml

a: oxidized corn starch.
b: 75-95 % Porcine gastric mucosa.
c: ≥ 20 % pectin from apples.
d: Salt concentrations were calculated from human fecal data; 130 – 150 mM Na^+ and K^+, 30 mM HCO_3^-(Rao 2011).
e: 8 g $CaCl_2$; 8 g $MgSO_4$ x 7 H_2O ad 1000 ml H_2O_{dest}
f: 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine x HCl, 50 mg thiamine x HCl, 50 mg Na-Riboflavin, 50 mg nicotinic acid, 50 mg Ca-Pantothenate, 1 mg vitamine B12, 50 mg 4-Aminobenzoic acid, 50 mg α-Lipoic acid ad 1000 ml H_2O_{dest} (Wolin et al. 1963). Sterile filtered.
g: ammonia concentration was calculated from human fecal data ~20 mM NH_4^+ (Wilson 1968; Wrong & Vince 1984; Eklou-Lawson et al. 2009, Wang et al. 2012).
h: 0.1 ml Vitamin K1 mixed with 20 ml 95 % ethanol
i: 50 % cholic acid sodium and 50 % deoxycholic acid sodium salt (Li et al. 2019).
j: 50 mg hemin mixed in 1 ml 1 M NaOH ad 100 ml H_2O_{dest}.

6.6 Cultivation of bacteria of the small and large Intestine

The organisms of the small and large intestine are cultivated anaerobically in modified SHIME® medium at 37 °C. Either cells are incubated under an N_2/CO_2 (80/20 %) atmosphere in serum flasks (100 ml) sealed with butyl rubber stoppers (Varel and Bryant 1974) or in a 48-well plate in an Infinite 200 PRO NanoQuant Microplate Reader (Tecan, Männedorf, Switzerland) under an N_2/CO_2 (80/20 %) atmosphere in an anaerobic chamber. For the latter purpose, the organisms are cultivated in 0.5 ml medium and growth behavior will be determined by measuring the optical density at 600 nm every 20 min, shaking the plate for 2 min before each measurement. Prior to inoculation, glucose, L-cysteine (0.5 g l^{-1}) as reducing agent, vitamin K1 (0.1% (v/v)) , vitamin solution (1 ml l^{-1}) and hemin (5 mg l^{-1}) are added. To test for the formation of API-NA, the respective API (see 6.4) and nitrate and nitrite are added to the modified SHIME medium (as indicated in section 6.3). After 24 h acetonitrile will be added and samples will be stored at -80 °C. For further investigations, kinetic studies will be performed by analysing API-NA formation hourly.

6.7 Determination of nitrite concentration

To evaluate whether the test organisms (Tab. 11) are able to reduce nitrate or nitrite 50 µl samples will be taken from each well as described above. If the organisms contain an active nitrate reductase we expect that the nitrite concentration will raise. This effect might contribute to the formation of API-NA. If the bacteria possess active nitrite reductases, NH_4^+ will be formed and the nitrite concentration declines thereby contributing to the detoxification of NO_2^- and preventing API-NA formation (please see introduction to section 6 for further explanation). The concentration of nitrite will be determined using the principle of *Lunge Reagent*. For the first solution (SA-solution), 1 g of sulfanilic acid is dissolved in 30 ml of glacial acetic acid and filled up to 100 ml with distilled water. For the second solution (NA-solution), 0.5 g naphthyl-1-amine is dissolved in 70 ml distilled water and 30 ml glacial acetic acid are added. By adding nitrite, the combination of both solutions produces an azo dye that has an absorption maximum at 540 nm (Zimmermann and von Lengerken 1979). With a nitrite standard (0-100 µM Na-nitrite) prepared in SHIME-medium and the measurement of the absorption at 540 nm, a calibration can then be carried out. In order to determine the concentrations of nitrite in the samples (6.6), 50 µl are taken and suspended solids are removed by centrifugation. The supernatant is mixed with 50 µl SA-solution and 50 µl Na-solution. After incubation for 10 min, the absorbance is detected at 540 nm. The concentration can then be determined with the use of the nitrite calibration.

7 QSAR calculations and in silico susceptibility of API for nitrosation

In order to make more precise statements on the nitrosability of chemical substances, especially drugs, in the future, models must be developed in which the results of in silico and in vitro/in vivo analyses are congruent. Important parameters are the physicochemical properties of the compound, such as the pk_a value. pk_a calculations were carried out in advance for some of the drugs included in the selection in order to estimate the degree of protonation of the secondary amines at a given pH value. This is very relevant for the reaction with nitrosyl cations. Within the study, however, other parameters for characterizing the drugs, such as steric hindrance, are to be calculated in order to draw conclusions about undesirable derivatization. The theoretically calculated results will be compared with the laboratory experimental results. ACD labs are used for the *in silico* analyses.

Table 17: Predicted values for all API compounds: pk_a values for the secondary amines or aniline (software ACD labs; Classic model) and ratio of protonated secondary amine / aniline summed up at the given pH value (software ACD labs; GALA model); high rates (green), low rates (red), medium (yellow).

Name_API	sec Amine pk_a (Base) (atom number)	Aniline pk_a (Base) (atom number)	sec Amine pk_a (Acid) (atom number)	sec Amine protonated [%]; ph 3	Aniline protonated [%]; ph 3	Sec. Amine protonated [%]; ph 7.4	Aniline protonated [%]; ph 7.4	CAS	API-derived NAs (Name)
Ephedrine	9.4 (11)	N/A	N/A	100	N/A	99	N/A	17608-59-2	N-Nitroso-ephedrine
Folic Acid	N/A	-0.63 (19)	15.78 (9)	0	4	0	0	29291-35-8	N-Nitroso-folic acid
Nortriptyline	10 (2)	N/A	N/A	100	N/A	100	N/A	55855-42-0	N-Nitroso-Nortriptyline

Hydrochlorothiazide	-4.08 (2)	N/A	N/A	0	N/A	0	N/A	63779-86-2	4-N-Nitroso-hydrochlorothiazide
Propranolol	9.5 (4)	N/A	N/A	100	N/A	100	N/A	84418-35-9	N-Nitroso-propanolol
Trimetazidine	9.07 (13)	N/A	N/A	100	N/A	95	N/A	92432-50-3	N-Nitroso-trimetazidine
Metoprolol	9.43 (4)	N/A	N/A	100	N/A	99	N/A	138768-62-4	N-Nitroso-metoprolol
Fluoxetine	10.05 (2)	N/A	N/A	100	N/A	100	N/A	150494-06-7	N-Nitroso-fluoxetine
Desloratadine (see Loratadine)	10.27 (14)	N/A	N/A	100	N/A	100	N/A	1246819-22-6	N-Nitroso-desloratadine
Rasagiline	6.95 (4)	N/A	N/A	100	N/A	61	N/A	2470278-90-9	N-Nitroso-rasagiline
Bumetanide	4.48 (5)	N/A	N/A	0	N/A	0	N/A	2490432-02-3	N-Nitroso-bumetanide
Duloxetine	10.2 (2)	N/A	N/A	100	N/A	99	N/A	2680527-91-5	N-Nitroso-duloxetine
Furosemide	-2.49 (7)	N/A	N/A	0	N/A	0	N/A	2708280-93-5	N-Nitroso-furosemide
Lorcaserin	9.99 (4)	N/A	N/A	100	N/A	99	N/A	2724616-80-0	N-Nitroso-lorcaserin
Varenicline	9.6 (4)	N/A	N/A	100	N/A	100	N/A	2755871-02-2	N-Nitroso-varenicline
Chloroquine	-3.24 (11)	N/A	N/A	0	N/A	0	N/A	nocas-3	N-Nitroso-chloroquine
Enalapril	5.43 (15)	N/A	N/A	100	N/A	1	N/A	nocas-5	N-Nitroso-enalapril
Salbutamol	9.62 (5)	N/A	N/A	100	N/A	98	N/A	nocas-9	N-Nitroso-salbutamol
Betahistine	9.75 (2)	N/A	N/A	100	N/A	100	N/A	32635-81-7	N-Nitroso-betahistine

8 Study outcome and readouts

The following tables and figures give an indication of how the results of the study will be presented and composed. At the beginning, the results will mainly consist of qualitative data. This includes, for example, the identification and characterization of the individual substances by LC-MS, in particular via MS and MS/MS experiments. For this purpose, an example MS/MS spectrum of atomoxetine and nitroso atomoxetine was selected and how the information obtained can subsequently be incorporated into an MRM experiment for quantification. The results of the validation are presented in a planned tabular format. The presentation of the quantitative conversions from the individual APIs to their nitroso derivatives will be done via a time and concentration dependent presentation. A decisive parameter will be the maximum yield of nitroso APIs produced within 60 minutes and thus also supplied to the intestine. For this purpose a heat map will be created. Furthermore, the calculation of corresponding rate constants as a function of the nitrite concentration and the pH value can be carried out under the assumption of certain reaction principles. The influence of food or nutrients and/or vitamin additives will also be recorded both graphically and in tabular form as a heat map in order to quickly identify and discuss the differences. Similarly, the results of the microbial study will be summarized, with characteristics of the bacterial strains, such as the availability of corresponding enzymes of the nitrite/nitrate metabolism, being discussed and evaluated in the results. In case no or only insufficient amounts of nitroso API are formed, possible decision guidance on how to deal with the results is summarized in table 20. This also explains possible perspectives for impacts in the future, open questions or suggestions for further actions.

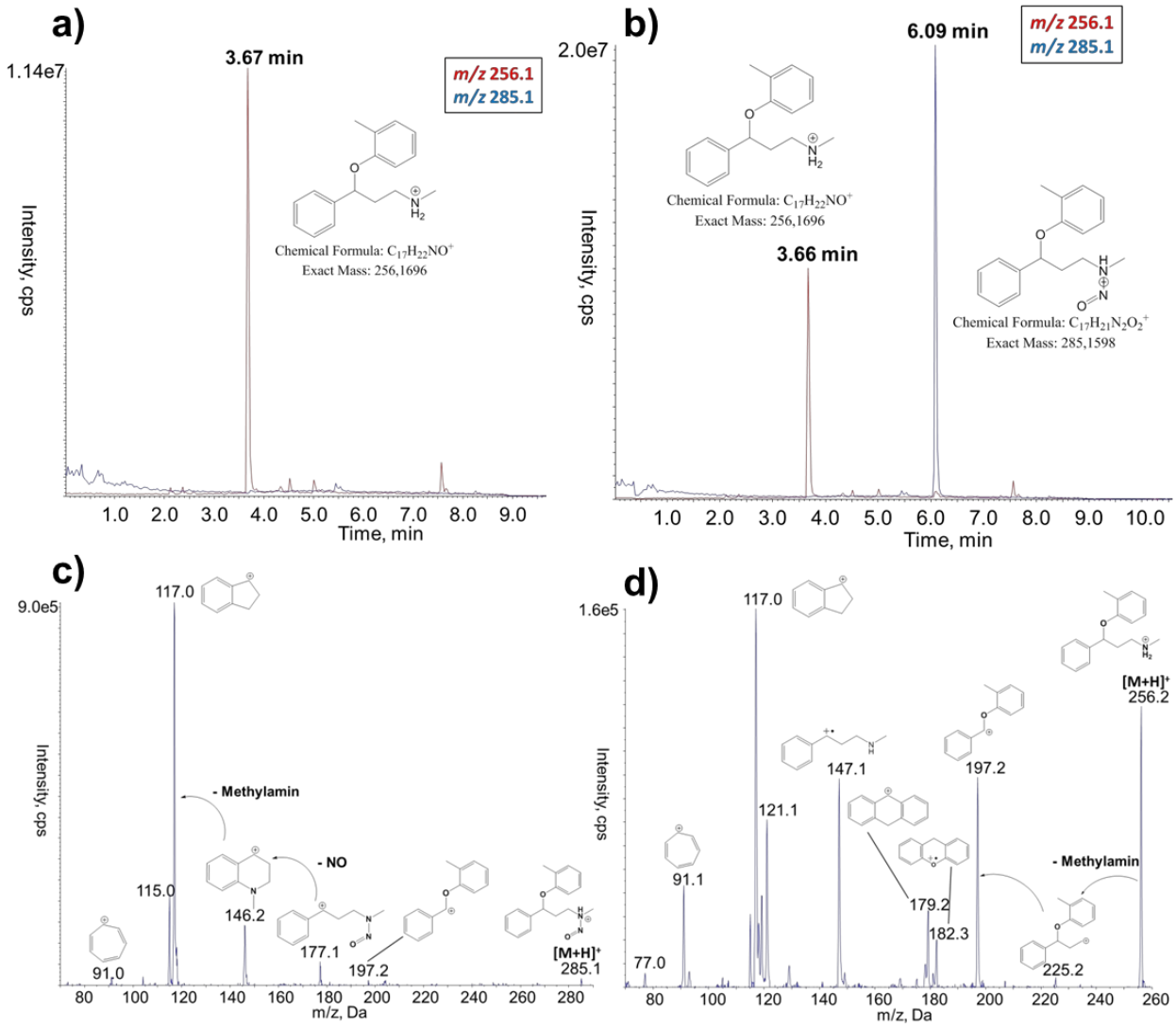


Figure 5: Enhanced product ion chromatograms from a) atomoxetin stock solution [1 µg/mL], b) atomoxetin stock solution [1 µg/mL] + 1 mg sodium nitrite (abs) + 2 µL hydrogen chloride solution [1 M], and c), d), their respective mass spectra with structure elucidation.

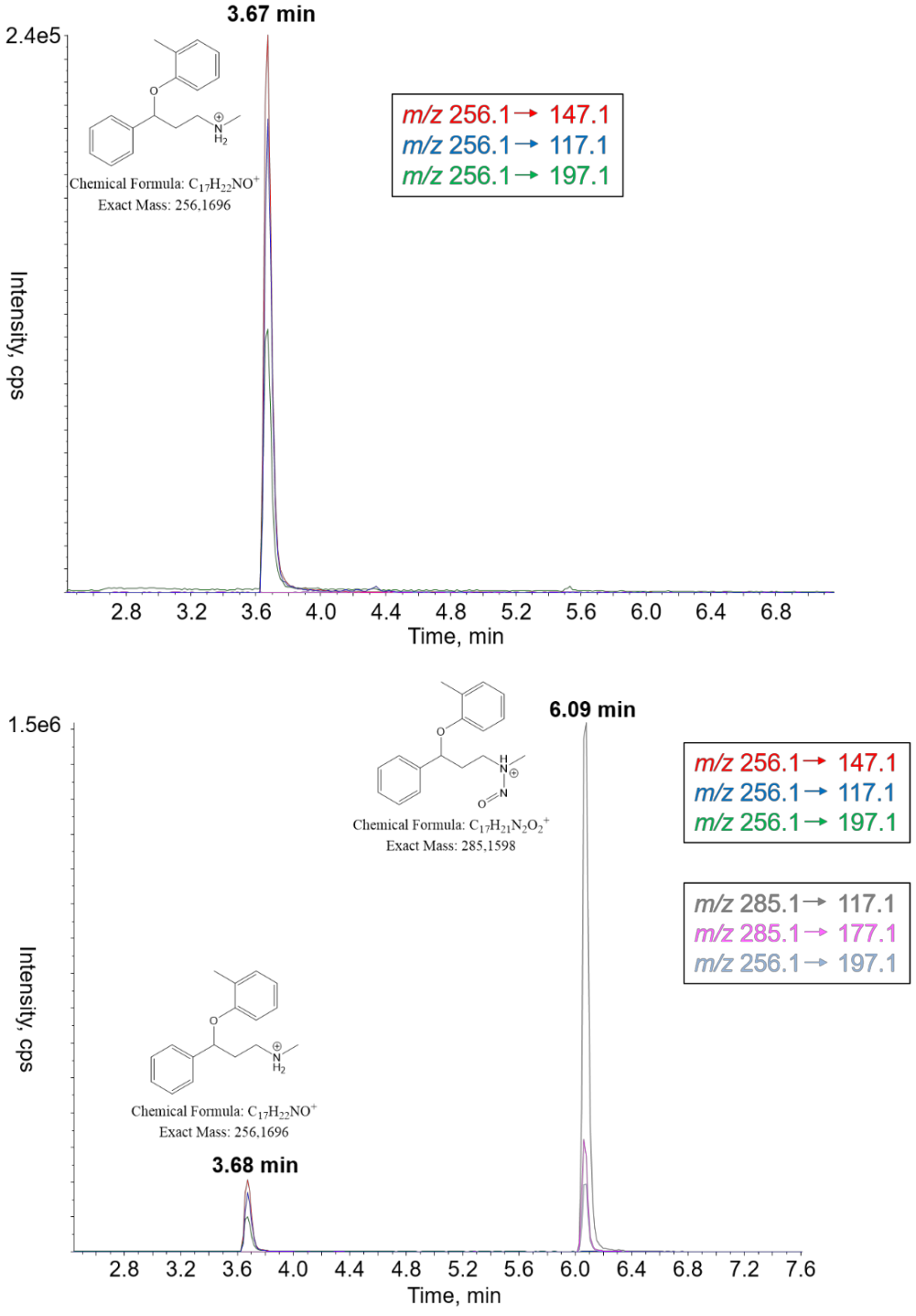


Figure 6: Multiple reaction monitoring (MRM) chromatograms for a) atomoxetin and b) atomoxetin derived nitrosoamine derivative

Table 18: Exemplary listing of validation results from Vogel et al. (Vogel and Norwig 2022)

ID	LOD [ppb]	LLOQ [ppb]	Linearity [R^2] / Range [ppb]			Repeatability [rel SD %]						Accuracy [%]						Recovery [%]	
	OES & AES	OES & AES	OES	AES	Range [ppb]	OES			AES			OES			AES			OES	AES
	[n=6]	[n=6]	[n=8]	[n=8]		5 ppb [n=6]	30 ppb [n=6]	150 ppb [n=6]	5 ppb [n=6]	30 ppb [n=6]	150 ppb [n=6]	5 ppb [n=6]	30 ppb [n=6]	150 ppb [n=6]	5 ppb [n=6]	30 ppb [n=6]	150 ppb [n=6]	30 ppb [n=6]	
NDMA	0.025	0.5	0.994	0.993	0.5 - 150	6.3	11.2	13.7	1.5	3.3	2.1	109.5	88.5	102.0	97.4	103.1	99.5	52.9	62.6
NMEA	0.15	1	0.99	0.998	1 - 150	8.3	11.9	12.7	9.2	7.7	3.3	102.1	88.1	113.3	101.8	100.2	100.4	45.1	50.3
NDEA	0.025	0.5	0.997	0.997	0.5 - 150	13.6	12.0	10.4	5.5	3.1	1.9	111.5	86.2	102.4	97.5	103.0	99.5	47.8	50.9
NDPA	0.15	1	0.996	0.991	1 - 150	14.7	5.8	9.6	8.8	9.3	10.7	104.3	94.8	101.1	94.4	106.7	98.0	58.3	66.1
NDBA	0.025	0.5	0.992	0.995	0.5 - 150	5.8	9.9	13.6	7.3	4.3	3.6	111.8	85.8	102.5	94.1	107.1	98.8	61.1	54.2
EIPNA	0.025	0.5	0.998	0.994	0.5 - 150	8.3	11.1	12.2	7.3	10.7	5.0	108.4	89.8	101.8	92.8	108.6	98.5	42.3	49.9
DIPNA	0.025	0.5	0.991	0.991	0.5 - 150	8.8	14.9	13.1	5.9	11.9	8.1	105.0	94.0	101.0	90.2	111.9	98.0	34.8	51.2
NPyr	0.75	5	0.993	0.999	5 - 150	7.1	8.4	10.8	3.1	8.0	2.6	102.2	83.8	114.0	102.8	96.6	100.6	28.0	33.4
NPip	1	5	0.996	0.991	5 - 150	14.6	14.2	9.6	8.8	8.4	2.9	101.2	91.3	107.5	94.8	106.3	98.9	34.1	33.3
NMor	0.025	0.5	0.996	0.991	0.5 - 150	14.3	14.4	13.5	13.7	13.4	4.7	111.9	91.4	102.5	95.5	110.6	99.1	53.6	59.2
NMBA	0.15	1	0.993	0.998	1 - 150	11.1	13.8	14.0	9.4	7.9	2.7	101.8	87.3	111.0	96.6	104.1	99.3	13.9	18.6
NMPA*	0.025	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NDPhA*	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Analytes failed validation; only LOD determined

	Intermediate Precision [rel SD %] OES									Intermediate Precision [rel SD %] AES								
	5 ppb [n=6]			30 ppb [n=6]			150 ppb [n=6]			5 ppb [n=6]			30 ppb [n=6]			150 ppb [n=6]		
	D1-D2	D1-D3	D2-D3	D1-D2	D1-D3	D2-D3	D1-D2	D1-D3	D2-D3	D1-D2	D1-D3	D2-D3	D1-D2	D1-D3	D2-D3	D1-D2	D1-D3	D2-D3
NDMA	2.3	2.6	0.3	5.6	2.4	3.3	0.6	1.2	1.7	4.8	5.8	1.0	1.5	1.0	0.5	1.6	0.6	0.9
NMEA	12.4	14.7	1.0	8.5	14.6	9.0	14.9	12.8	4.7	2.0	8.6	6.6	3.7	10.0	6.3	6.8	11.6	4.9
NDEA	8.4	0.5	8.9	1.3	2.8	6.2	1.8	1.8	0.1	2.9	3.2	0.2	0.5	0.2	3.1	3.0	0.2	3.1
NDPA	4.4	6.6	11.0	13.1	14.9	13.8	0.3	10.4	10.8	4.7	5.9	6.8	7.0	11.5	12.5	3.3	13.9	10.6
NDBA	0.9	4.8	3.9	2.3	3.2	0.8	3.4	7.8	4.5	0.2	2.5	2.3	11.5	8.0	3.5	7.3	4.4	2.9
EIPNA	8.7	0.2	8.5	3.4	13.1	14.1	7.5	3.0	10.5	12.7	4.4	13.1	13.6	3.7	12.5	11.9	1.5	13.2
DIPNA	6.8	1.0	7.8	5.5	7.0	12.5	4.2	0.1	4.2	13.2	13.9	10.4	7.1	2.3	0.8	14.2	13.8	5.9
NPyr	13.1	14.5	0.5	9.7	11.0	1.3	1.4	1.2	0.1	5.3	14.1	13.1	1.4	8.6	10.0	5.5	2.0	3.4
NPip	6.3	13.0	10.7	7.2	6.9	0.3	1.5	7.8	6.3	11.9	13.8	4.1	13.5	13.7	6.1	12.4	14.9	3.6
NMor	0.9	0.6	0.3	6.8	7.3	0.5	1.3	3.8	5.1	13.6	14.5	0.9	4.5	0.7	5.2	7.6	2.6	5.0
NMBA	9.9	4.4	5.5	13.3	0.2	13.1	1.2	10.2	13.3	4.6	4.7	9.3	2.2	2.3	4.5	10.1	5.5	3.0

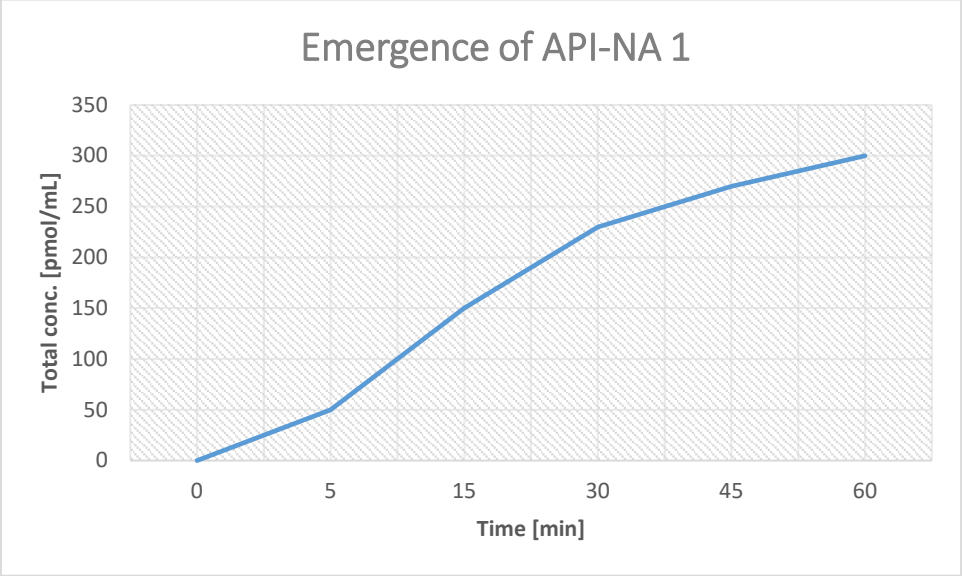


Figure 7: Exemplary representation of the formation kinetics of an API-NA at a certain pH and nitrite concentration.

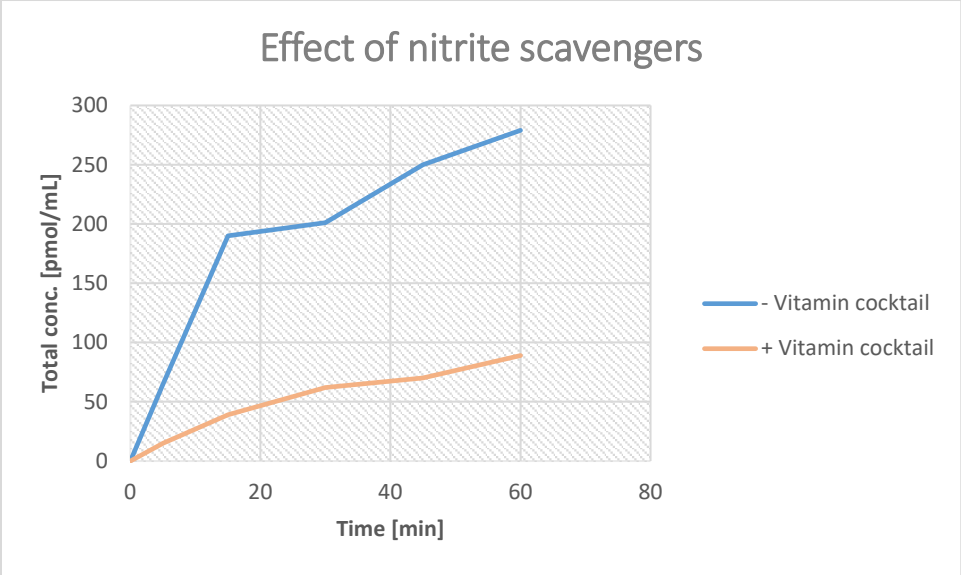


Figure 8: Exemplary presentation of the influence of the use of nitrite scavengers at a certain pH value and nitrite concentration on API-NA formation.

Table 19: Exemplary graphical heat map of the totally emerged amount of API-NA evaluated relative to each other.

pH / Nitrite conc.	pH 1.5	pH 3.15	pH 4	pH 6	pH 7.4
1 µM	Quantitative total amount of API-NA after 60min				
33 µM					
200 µM					
1 M					

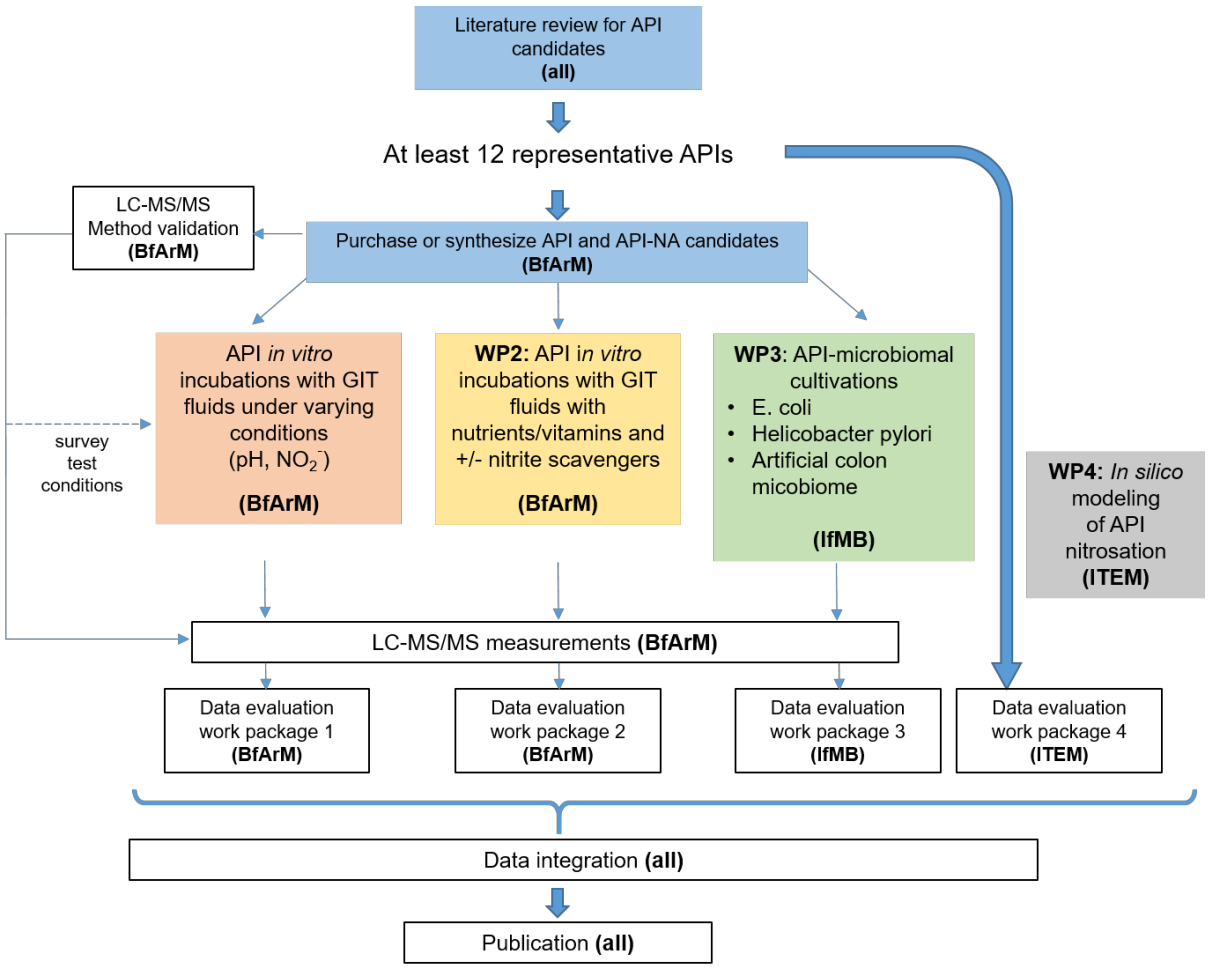
Table 20: Summarized expected outcomes and related readouts for the main experimental parts of the study.

Part of the study	Outcome	Readout
Analytical method development and validation	Proof-of principle compound identification by means of LC-MS	• Implementation of LC-MS methods for future applications • Obtaining essential device settings • APCI or ESI ion source suitability?
	Highly sensitive determination of API-NA; LOD/LOQ	• Implementation of threshold values for system suitability • Method improvement of the corresponding nitroso derivatives across drug classes and selection of appropriate device settings
	Successfully passed validation	• Publication of the final method and listing of analytical pitfalls and challenges • Adoption of the method in other laboratories
In vitro gastric incubations	**Positive** qualitative and quantitative determination of API-NA derivatives	Key points: • Data collection and root-cause analysis for non-conversion • Define nitrosation tendencies for similar API or API classes •
	Negative qualitative and quantitative determination of API-NA derivatives	Key points: • Calculation and summary of the kinetics of formation • Identification of the responsible microbiota in the different gut sections • Investigation of accompanying nitrite forming reactions via bacteria •
In vitro intestinal incubations	**Positive** qualitative and quantitative determination of API-NA derivatives	Key points: • Cause research for non-conversion • Investigation of accompanying detoxifying reactions via bacteria

		• Investigation of metabolic deactivation of nitrite via bacteria
	Negative qualitative and quantitative determination of API-NA derivatives	• Scientific publication of the *in silico* model for future calculations • Basis as an assessment aid for other drug substances
QSAR calculations	**Agreement** of QSAR and laboratory experimental results	• Performing further calculations to identify possible unconsidered physicochemical parameters • Discuss deviations in the scientific publication
	Non-agreement of QSAR and laboratory experimental results	•

9 Appendices

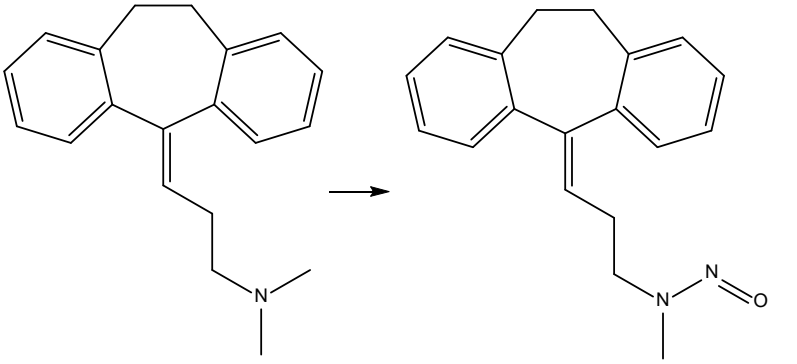
9.1 Responsibilities of the study



9.2 Monographs final compounds

The compounds are sorted alphabetically. Most of the compounds are commercially available with more or less delivery time. The assessed pk_a values were reviewed for relevant amines of the molecular structure, as well as amines which undergo nitrosation.

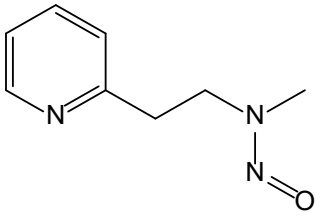
- **Amitriptyline / N-Nitrosonortriptyline (CAS 55855-42-0)**



Amitriptyline *N*-Nitrosonortriptyline

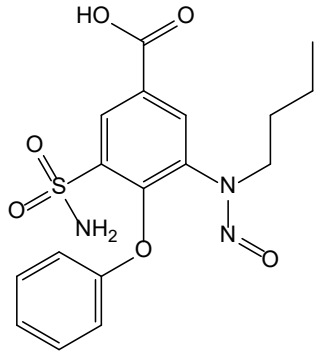
Rationale: Amitriptyline is a first generation tricyclic antidepressants. In reference to Ziebarth (1985) a nitrosative dealkylation was observed under simulated gastric conditions followed by the release of NDMA. This chemical reaction raise the potential for further reactive processes and/or the emergence of the degradation product nitrosonortriptyline.

- **Betahistine / N-Nitrosobetahistine (CAS 32635-81-7)**



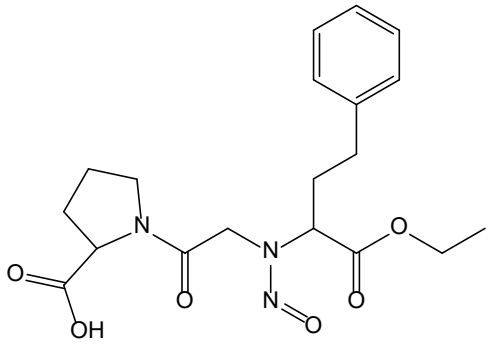
Rationale: According to the publication of Schmidtsdorff *et al.* Betahistine has a high nitrosation rate with x>99.9% after nitrite treatment, making the compound adequate as benchmark (Schmidtsdorff, Neumann et al. 2022). Additionally, the adjacent residues to the nitroso group are of interest, due to the conceivable release of highly toxic methyl carbenium ions.

- **Bumetanide / N-Nitrosobumetanide (CAS 2490432-02-3)**



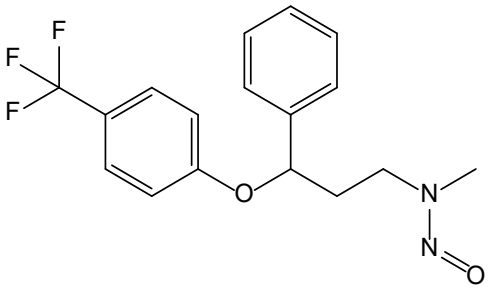
Rationale: Bumetanide can be seen as nitroso-butylphenyl derivative substituted with acidic groups. In case of alpha hydroxylation in the butyl side chain, and further release of phenylic carbenium ion, mesomeric stabilization can be expected as a matter of the latter acidic moieties. Many API contain acidic substituted phenylic systems. Therefore, bumetanide seems to be a representative candidate to investigate the reactivity and stability of these kind of positively charged ions.

- **Enalapril / N-Nitrosoenalapril (CAS N/A)**



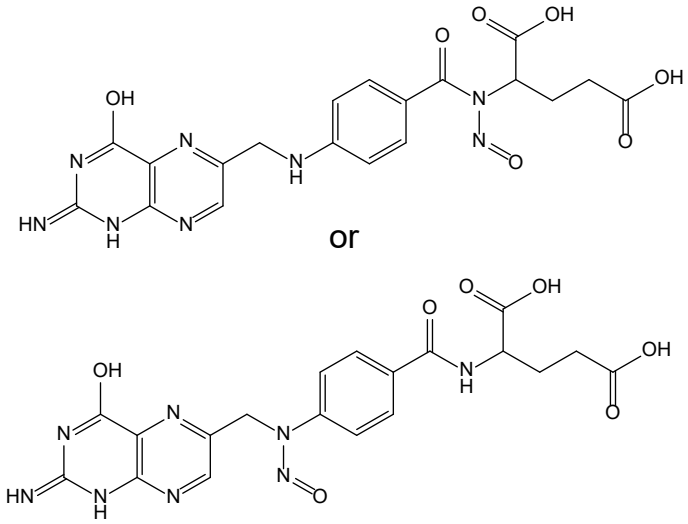
Rationale: Prior reports show a good nitrosation yield with 67.8 % under artificial conditions (Schmidtsdorff, Neumann et al. 2022). Genotoxic activities were also investigated from Ozhan and Alpertunga (Ozhan and Alpertunga 2003). More carcinogenic evidence was also found in the proposition 65 ‘Evidence on the Carcinogenicity of Nitrite in Combination with Amines or Amides’ from the ‘Reproductive and Cancer Hazard Assessment Branch Office of Environmental Health Hazard Assessment California Environmental Protection Agency’ from 2016 (OEHHA 2016).

- **Fluoxetine / N-Nitrosofluoxetine (CAS 150494-06-7)**



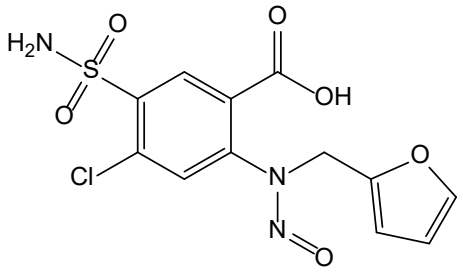
Rationale: Additionally, similar to Betahistine, Fluoxetine bears a high nitrosation rate (Schmidtsdorff *et al.*, x>99%) and representative side chains, in particular a more bulky fluorinated aromatic chain and a methyl residue, whose alkylation tendency and mutagenic effects are well investigated (Schmidtsdorff, Neumann et al. 2022). It is genotoxic to S. typhimurium when used at concentrations ranging from 0.06 to 0.12 mg/mL (Ozhan and Alpertunga 2003).

- **Folic Acid / N-Nitroso folic acid (CAS 29291-35-8 for amide, CAS 26360-21-4 for primary aromatic amine)**



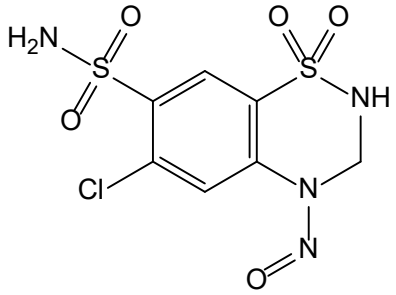
Rationale: Folic acid contains two conceivable reactive chemical groups for nitrosation. However, in the case of nitrosation, the secondary aromatic amine is of greater importance. Another important point is the large spatial expansion of the molecule, which makes it interesting as a model substance. Relevant links belonging toxicological data were found on PubChem (PubChem 2022). Carcinogenicity research for nitroso folic acid was performed in mice (Wogan, Paglialunga et al. 1975).

- **Furosemide / N-Nitrosofurosemide (CAS N/A)**



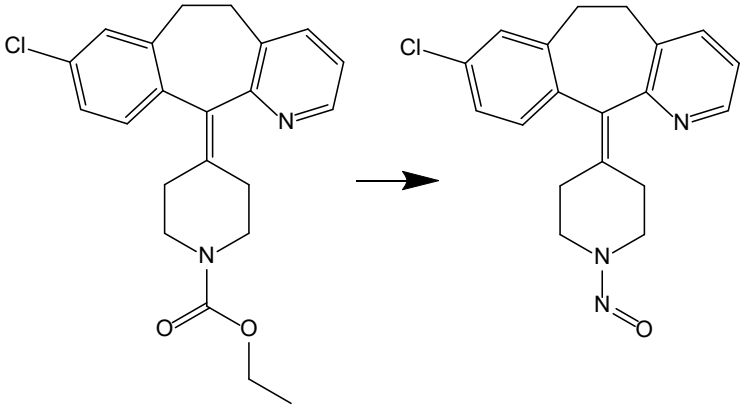
Rationale: Analysis of nitrosofurosemide and the respective nitrosation rate of furosemide was conducted by Brienza *et al.* (2019) under UV atmosphere for wastewater treatment (Brienza, Manasfi et al. 2019). Approaches for nitrosofurosemide occurrence under physiological conditions have not yet been performed. Nitrosation tendencies were described by Brambilla in 2007 and lay between 50-52% (Brambilla and Martelli 2007). First reports of the occurrence in susceptible APIs, also in furosemide was published in 1985 (Brambilla, Cajelli et al. 1985).

- **Hydrochlorothiazide / N-Nitrosohydrochlorothiazide (CAS 63779-86-2)**



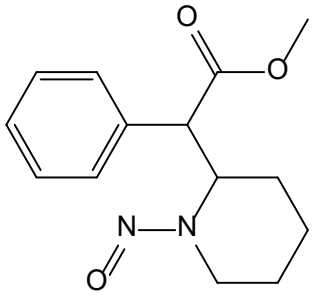
Rationale: Nitroso-HCT is a well investigated molecule and publications were released Brienza *et al*., Gold *et al.* and Lijinsky *et al* (Gold and Mirvish 1977, Lijinsky and Reuber 1987, Brienza, Manasfi et al. 2020). All three publications confirm the occurrence of Nitroso-HCT in the presence of nitrite. Furthermore, a low nitrosation tendency of HCT of 19.2% under forced degradation, was recently published (Schmidtsdorff, Neumann et al. 2022). IARC lists Nitroso-HCT as possible mutagenic compound (IARC 2022). Another investigation was performed in very close relation to the upcoming experiments by Gillatt *et al.* (Gillatt, Palmer et al. 1985). In this study, artificial gastric conditions were used to mimic nitrosation of APIs *in vitro*.

- **Loratadine/N-Nitroso desloratadine (CAS 1246819-22-6)**



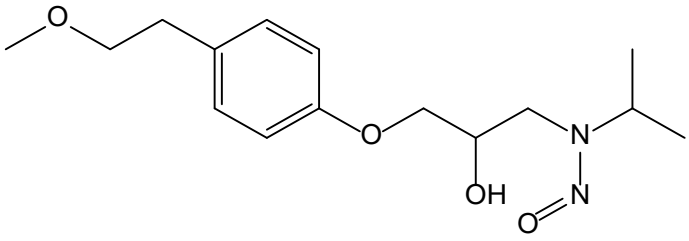
Rationale: The rationale behind choosing Loratadine as adequate molecule is reasoned in its form as a predrug and the question if protected secondary amines are susceptible towards nitrosation under physiological conditions. The instable carbamate is well established as chemical predrug group. Obtained results concerning the instability and susceptibility of nitrosation can be used to estimate another carbamate protected. Schmidtsdorff *et al.* determined the nitrosation rate with a fourfold molar excess of nitrite with 29.2%(Schmidtsdorff, Neumann et al. 2022).

- **Methylphenidate / N-Nitrosomethylphenidate (CAS 55557-03-4)**



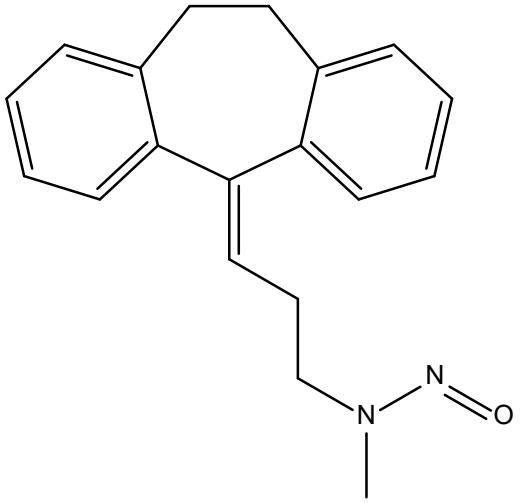
Rationale: Against first results of Giner-Sorolla et al. and Lijinsky et al., which determined no carcinogenic effects of nitroso-methylphenidate in rodents, the compound emerges in literature frequently and was also part of bacterial mutagenicity tests(Lijinsky and Taylor 1976, Rao, Hardigree et al. 1977, Giner-Sorolla, Greenbaum et al. 1980). Additionally, computer assisted studies were performed to calculate structure-activity relationships(Rose and Jurs 1982).

- **Metoprolol / N-Nitrosometoprolol (CAS 138768-62-4)**



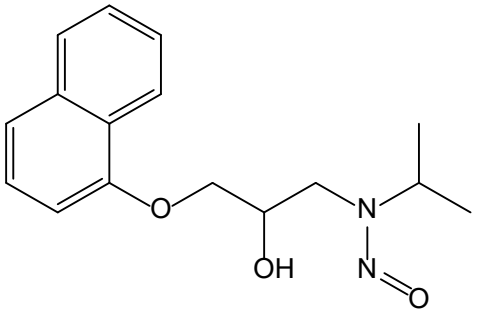
Rationale: The isopropyl group can form highly reactive isopropyl cations after alpha hydroxylation. Additionally, there is already a hydroxy function at the beta carbon atom. Beta hydroxylations adjacent to nitroso groups are discussed to incorporate mutagenic potential after metabolism(Teiber, Mace et al. 2001). Publications investigating Nitrosometoprolol were also multiply published by Martelli, Robbiano and Brambilla, and Schmidtsdorff (2022)(Robbiano, Martelli et al. 1991, Martelli, Allavena et al. 1994, Martelli 1997, Schmidtsdorff, Neumann et al. 2022). The latter revealed a nitrosation rate of metoprolol under forced conditions with approximately 50%.

- **Nortriptyline / N-Nitrosonortriptyline (CAS 55855-42-0)**



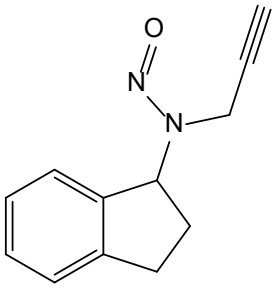
Rationale: see Amitriptyline

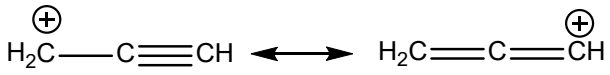
- **Propranolol / N-Nitrosopropanolol (CAS 84418-35-9)**



Rationale: Propranolol is very close to metoprolol and similar toxicological properties can be expected after nitrosation and metabolization. Although, the *in vitro* nitrosation rate of propranolol is approximately 19% and therefore lower as metoprolol, but the availability of data with regards to nitrosation rates in the gastrointestinal tract is highly evident (Chen and Raisfeld-Danse 1983, Raisfeld-Danse and Chen 1983, Gillatt, Palmer et al. 1985, Robbiano, Martelli et al. 1991, Martelli, Allavena et al. 1994, Brambilla, Martelli et al. 1995, Sottofattori, Anzaldi et al. 1997, Sottofattori, Martelli et al. 2001). The latter publications also contain analytical methods and QSAR approaches.

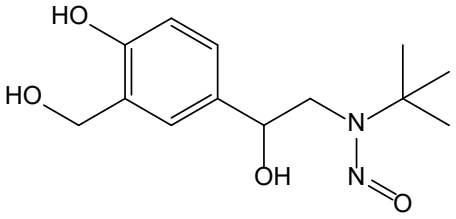
- **Rasagiline / N-Nitrosorasagiline (CAS 2470278-90-9)**





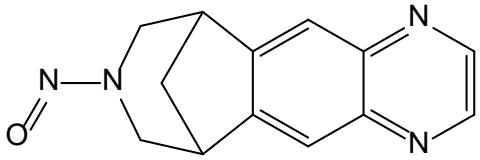
Rationale: This relatively small API can form mesomeric stabilized carbenium ions for both residues. Thereby, the propynylium cation after toxification is of high interest, as it comprises low molecular space and mesomeric stabilization. Reports were only found within patent documents on rasagiline impurity preparation methods and applications (Ma, Yuntao; Wei, Ran; Li, Wan; Mu, Yongyue; He, Xianliang; Huang, Luning; Tao, Anping; An, Jianguo; Gu, Hong China, CN113045456 A 2021-06-29)

- **Salbutamol / N-Nitrososalbutamol (CAS N/A)**



Rationale:. Salbutamol is considered to lead to tertbutyl-cation with lower reactivity towards isopropyl-cation (see Metoprolol, Propranolol). Nevertheless, toxicity and alkylation tendency of tert butyl cations should be evaluated to corroborate lower reactivity and further provide new data to assess mutagenic risk. In the past, Nitrososalbutamol containing medicines were withdrawn from the market (Recall of Ventolin® (GSK) (SingaporeHealthSciencesAuthority 2021).

- **Varenicline / N-Nitrosovarenicline (CAS N/A)**



Rationale: Varenicline is a polyaromatic compound with an additional bicyclic ring system. Vareniclin was chosen as representative test candidate due to its unique and dense ring systems. Within the publication of Schmidtsdorf, Varenicline revealed a nitrosation rate of 69%(Schmidtsdorff, Neumann et al. 2022).

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