Title: Limits for *N*-Nitrosamines in Drug Products and their Structure-Activity Relationship
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Threshold Limits for *N*-nitrosamines

Based on the *Threshold of Toxicological Concern (TTC)* concept described in ICH M7 (1), most mutagenic impurities are limited to a dose of 1.5 µg/day which corresponds to a 10^{-5} excess lifetime risk of cancer.

However, *N*-nitrosamines belong to a class of substances that are highly carcinogenic at very low concentrations. This group of substances is called *"cohort of concern"* and has been defined in ICH M7. The group comprises aflatoxin-like, *N*-nitroso, and alkyl-azoxy compounds. Substances belonging to the cohort of concern expose patients to a significant carcinogenic risk at levels far below the general TTC of 1.5 µg/day. Based on substance specific TD_{50} values, limits for nitrosamines are to be set in accordance with the ICH M7, so that the patient risk levels do not exceed 1 in 100,000.

Tumor dose 50 (TD_{50}) is the daily dose causing tumours in 50% of animals in a lifetime. To determine the TD_{50} usually long-term carcinogenicity studies are conducted on rodents. These TD_{50} values can be used to set acceptable intake (AI) levels for humans (1). A TD_{50} of 1.5 mg/kg/day corresponds to a human lifetime AI of 1.5 µg/day.

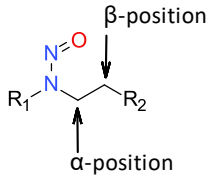
According to the European Medicines Agency a class-specific threshold of toxicological concern of 18 ng/day can be used if no robust carcinogenicity data are available (2). The European Regulatory Network approach (2) also opens the possibility to define *acceptable intake (AI) levels* based on *structure-activity relationships (SARs)*.

Structure-Activity Relationship

The possibility to adjust AI levels based on SAR is particularly helpful: Small *N*-nitrosamines like *N*-Nitrosodimethylamine (NDMA) and *N*-Nitrosodiethylamine (NDEA) are typically potent, falling in a narrow TD_{50} range, whereas the group of *N*-nitrosamines, including drug-like substances, shows TD_{50} values ranging from 0.01 mg/kg/day to over 100 mg/kg/day (3). This corresponds to acceptable intakes from < 10 ng/day to > 100 ng/day. Therefore, the group of *N*-nitrosamines also includes substances with much lower potency compared to NDMA.

According to Cross and Ponting (3), there are at least three structural factors that can influence the toxicity of N-nitrosamines. These are:

1) The presence of an α-proton
2) Steric hindrance at the α-carbon, and
3) The presence of electron-withdrawing groups with effect on the α-position, such as $-NO_2$, -CN, CF_3 in α position or C=O in β-position



N-nitrosodialkylamine

The first two factors are closely related as a tertiary carbon atom in α-position will have no proton left whilst being sterically hindered. The effects can be understood by looking at the predominant metabolic pathway (**Figure 1**) (3). The highest potency *N*-nitrosamines follow this pathway for which the α-carbon is hydroxylated in a first step by P450 isozymes, making the availability of one proton in the α-position essential. Arguably, if no α-proton is available the compound should not be considered to belong to the cohort of concern. The compound lacks the extraordinarily high toxicological

potential of this group (1,3). For small dialkylnitrosamines, the α-hydroxylation is catalysed predominantly by CYP2E1 and CYP2A6 (4). The active site of CYP1E1 is reported to be small, explaining the effect of reduced toxicity when sterically hindering groups are present at the α-carbon. However, other isoforms have been reported to be involved in the α-hydroxylation of nitrosamines, also allowing the activation of larger nitrosamines (CYP3A4) or substrates with anionic sites or steric requirements (CYP2C9) (5).

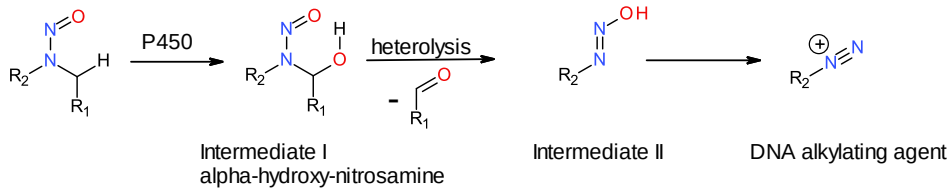


Figure 1: Predominant metabolic pathway of highest potency nitrosamines, adapted from Cross and Ponting (2021) (3).

The presence of one tertiary butyl group in α-position eliminated the carcinogenicity in experiments, whilst the presence of one or two iso-propyl or aryl groups only reduced toxicity. Therefore, nitrosamines like the structures in **Figure 2** of *N*-nitroso salbutamol and *N*-nitroso vildagliptin can be expected to be less toxic than NDEA or NDMA.

Figure 2: N-nitrosamines with sterically hindered α-positions.

Reliable data on carcinogenicity can be found in the [Lhasa Carcinogenicity Database] (6).

Indapamide related compound D (**Figure 3**) contains an isopropyl-like moiety and an aryl group in α-position. Both substituents should reduce toxicity due to steric hindrance (iso-propyl) or absence of an α-proton (aryl). However, there remains the risk that this compound is still toxic because both groups do not seem to fully eliminate toxicity. The compound is currently limited to 5 parts per million (ppm) within the Indapamide EP monograph (EP 9.2).

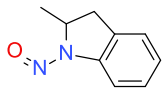


Figure 3: Only very bulky α-substituents eliminate toxicity, Indapamide related compound D is limited to 5 ppm (EP 9.2).

Cross and Ponting (3) also showed that strong electron-withdrawing groups (EWG) with effect on the α-position can reduce carcinogenicity, which is likely a result of the reduced electron density that makes the oxidation less favourable. These findings are in line with Mesic *et al.* (7), suggesting that the stability of intermediate I is reduced by the presence of an electron-withdrawing group.

Only strong EWG like -CF_3, -CN, or -NO_2 clearly show a decreasing effect on carcinogenicity.

However, the effects of weak EWG like carbonyl groups or aromatic systems remain difficult to predict.

In this article only the carcinogenicity of nitrosamines formed from secondary alkylamines has been discussed so far. However, there are a number of substance classes (***Figure 4***) which may have substantially different pathways of developing their toxicity. When setting limits for these kind of *N*-nitrosamines by read-across from known carcinogens, it is important to compare compounds of the same substance class.

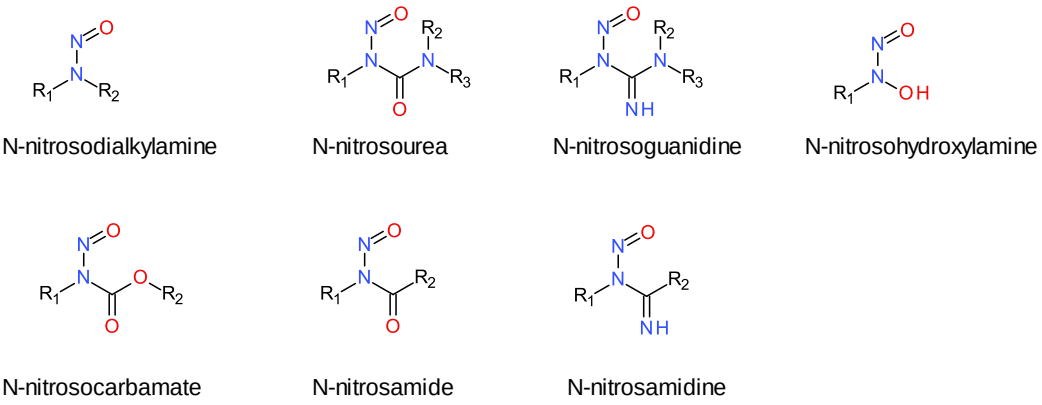


Figure 4: Structural classes of N-nitroso compounds.

N-nitrosoureas for example are unstable and form the DNA alkylating agent directly via degradation instead of the above shown α-hydroxylation by P450s. *N*-nitrosoureas are direct alkylating agents which quickly undergo hydrolysis under basic conditions as described by Golding *et al.* (8).

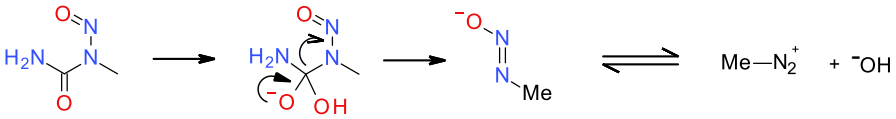
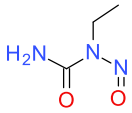
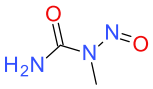


Figure 5: Decomposition of N-nitrosoureas.

Therefore, these compounds are always carcinogenic, developing their toxicity depending on availability and distribution *in vivo*. *N*-Nitroso-*N*-ethyl urea (ENU) and *N*-Nitroso-*N*-methyl urea (NMU) (**Figure 6**) are both highly potent carcinogens and mutagens, NMU having a Lhasa TD_{50} in mice of 0.803 mg/kg/day.



N-nitroso-N-ethyl urea
ENU



N-nitroso-N-methyl urea
NMU

Figure 6: Highly carcinogenic ureas ENU and NMU.

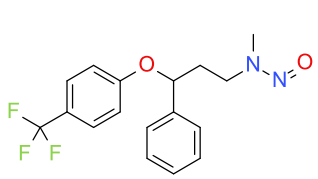
Conclusion:

For *N*-nitrosamines a class-specific threshold of toxicological concern of 18 ng/day can be used if there is not sufficient robust carcinogenicity data to define a compound-specific limit. This limit can be amended based on a well-established structure-activity relationship, which can be used to define a suitable structural analogue with reliable carcinogenicity data. The potency of a sufficiently close analogue can be assumed to be comparable. For *N*-nitrosodialkylamines, branching at the α-position is the most important factor which determines toxicity next to the presence of strong electron-withdrawing groups. When justifying acceptable intake limits by using carcinogenicity data from structurally related compounds in a read-across assessment, it is important to consider the substance classes of *N*-nitrosamines as they can have different pharmacokinetic profiles.

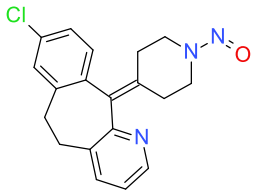
LGC. Science for a safer world

LGC Standards is committed to providing the reference materials needed to produce high-quality, safe medicine. Relevant *N*-nitrosamines are added regularly to an extensive portfolio, so the correct reference materials are at hand for identification and quantification of impurities. The latest of these additions to LGC's Mikromol reference materials range are highlighted in **Figure 7** .

Fluoxetine is a very important antidepressant and desloratadine is a commonly used antihistamine for treating allergies. Both nitrosamines should be expected to be highly carcinogenic. For *N*-nitroso desloratadine, it may be possible to establish a limit based on carcinogenicity data available for *N*-nitroso-4-piperidone (9), which is structurally closely related. Both *N*-nitrosamines therefore require accurate controls in drug substances and drug products that can be established by use of suitable reference standards.



N-nitroso fluoxetine
CAS: 150494-06-7
MM0256.22-0025



8-Chloro-6,11-dihydro-11-(1-nitroso-4-piperidinylidene)-5H-benzo[5,6]cyclohepta[1,2-b]pyridine
CAS: 1246819-22-6
MM0257.25-0025

Figure 7: New N-nitrosamine additions to the Mikromol reference materials catalogue.

If you are looking for a specific* N*-nitrosamine to confirm absence of nitrosamines in question, or for quantification, for AMES testing, or rodent carcinogenicity tests, please contact the LGC product management team at Pharmaservices@lgcgroup.com.

Acknowledgements:

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8. B. T. Golding et al., Tetrahedron **53**(11), 4063–4082 (1997)

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