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Improved Mutagenicity Testing of Nitrosamines Using Miniaturized Ames Assays

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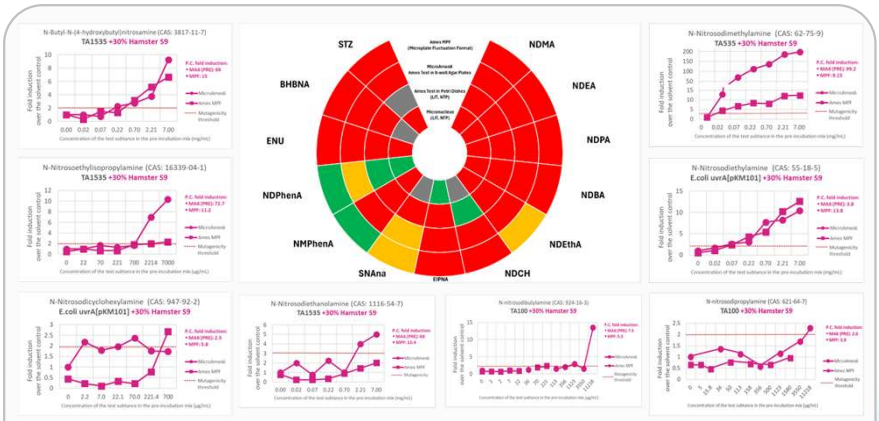
Background

Detecting **genotoxic impurities in pharmaceuticals** is critical to ensuring drug safety and protecting public health. Rising concerns over N-Nitrosamines - potent carcinogens found in some drug products - have driven global efforts to improve testing methods that can efficiently address the mutagenicity of substances in this chemical class. The mechanisms through which nitrosamines induce genotoxicity include DNA alkylation, oxidative stress induction, and adduct formation. **Enhanced sensitivities** have been achieved through qualitative and quantitative Ames Test analyses, pinpointing effective experimental conditions for **accurate mutagenicity detection** [1]. Considerable concerns have emerged surrounding the presence of these substances in pharmaceuticals, with contaminants potentially leading to critical safety issues. Recent testing and reporting guidance by the FDA highlighted the issue of **leachable small-molecule Nitrosamines** found in drug products packaged in infusion bags [2]. The FDA and EMA have heightened scrutiny of manufacturing processes to mitigate nitrosamine formation. The regulatory recommendation includes the application of the **Enhanced Ames Test (EAT)** principle with the recommendation to use **30% Hamster Liver S9** in addition to Rat Liver S9 [3,4].

Materials and Methods

In this study, we applied two miniaturized versions of the Ames assay to evaluate the mutagenic potential of N-Nitrosamines. The first was the **MicroAmes6**, an agar-based assay conducted in a 6-well plate format following the pre-incubation protocol. The second was the **Ames MPF**, an Ames assay in microplate fluctuation format. Both miniaturized assays were adapted to use the following **OECD TG 471-compliant** Ames tester strains: Salmonella typhimurium strains TA100 and TA1535, as well as Escherichia coli uvrA[pKM101]. These Ames tester strains are recommended by the regulatory authorities for the characterization of N-Nitrosamine mutagenicity. To enable metabolic activation - a critical process when testing pro-mutagens such as many of the N-Nitrosamines - we implemented the **Enhanced Ames Test conditions** in both miniaturized formats. 30% Hamster Liver S9 mix was used, selected for its superior metabolic capability in bioactivating N-Nitrosamines compared to rat liver S9 microsomal fraction. The protocols were refined to accommodate a wide range of N-Nitrosamine compounds, including **both volatile and non-volatile** substances. These optimizations were aimed at maximizing assay sensitivity and reliability for detecting mutagenicity in structurally diverse N-Nitrosamines.

Results



Selected experimental results from the study showing the performance of the miniaturized Ames assays | Fold induction over the solvent control in the number of revertant wells or revertant colonies is presented as the function of the varying concentration of the Nitrosamine test substances, for Ames MPF and for MicroAmes6, respectively. Dashed red line is the threshold for positivity. Circles and squares with dotted pattern represent cytotoxic concentrations. Concurrent positive control fold induction values are indicated next to the graphs. MA6 = MicroAmes6, Ames assay in 6-well agar plate format; MPF: Ames test in microplate fluctuation format; PRE: pre-incubation protocol. **Summary of the study and comparison of the miniaturized Ames test results with Petri dish-based Ames test data and micronucleus data from the NTP database [5] or scientific literature** | For the miniaturized Ames assays we present cumulative test result, i.e. a compound with one positive result in one Ames tester strain is assessed as a positive compound. The compound is negative if it is tested negative with the miniaturized Ames assays in all three tester strains. The compound is equivocal if it is tested equivocal in the miniaturized Ames assays with at least one Ames tester strain, but all other strains are negative. For the Petri dish-based Ames test, the cumulative call was based on all available results published in the NTP database [5] or in the literature. In vitro or in vivo micronucleus test results for the corresponding Nitrosamines were collected from the NTP database [5]. Color codes: Red = positive, yellow = equivocal, green = negative, grey = not available.

Conclusion

This study highlights the value of **miniaturized Ames assay formats** in addressing the challenges of detecting **genotoxic impurities**, particularly **Nitrosamines**, at **low concentrations**. While the traditional Petri dish-based Ames assay is a proven method, it is resource-heavy and not optimized for **complex mixtures** or **packaging-related leachables**. By contrast, miniaturized formats such as **Ames MPF** and **MicroAmes6** reduce reagent use and test material requirements, while enabling high-throughput screening of multiple compounds and mixtures in parallel. These formats not only streamline workflows but also retain - and in some cases enhance - **sensitivity**, allowing for reliable detection of Nitrosamines and other genotoxic contaminants. In our study, both Ames MPF and MicroAmes6 consistently identified mutagenic responses of Nitrosamines, including levels relevant to impurities that may migrate from **packaging materials** or arise in **complex formulations**.

References:
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[3] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry
[4] European Medicines Agency. (2023, July). Questions and answers for marketing authorisation 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
[5] National Toxicology Program (NTP) coordinated by United States Department of Health and Human Services