Nitro Musk Compounds

Genotoxic Activity

Genotoxicity Testing of Nitro Musks with the SOS-Chromotest and the Sister-Chromatid Exchange Test

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Abstracts

Five nitro musk compounds are widely used as fragrance ingredients in perfumes, lotions and detergents; as food additives in cigarettes and fish baits, and in such technical products as herbicide formulations and explosives. Several studies identified nitro musk compounds in aquatic environment samples, human milk and fat samples as highly lipophilic and persistent bioaccumulating environmental pollutants. To examine the compounds for genotoxic activity, musk xylene (1-tert.-butyl-3,5-dimethyl 2,4,6-trinitrobenzene), musk ketone (4-tert. butyl-3,5-dinitro-2,6-dimethylacetophenone), musk ambrette (1-tert.-butyl-4-methyl-6-methoxy-3,5-dinitrobenzene), musk moskene (1,1,3,3,5-pentamethyl-4,6-di-nitromdane) and musk tibetene (1-tert.-butyl-3,4,5-trimethyl-2,6-dinitrobenzene) were tested for SOS inducing potency in the SOS chromotest with E. coli PQ37 and for sister-chromatid exchange inducing activities in human lymphocytes in vitro both in the presence and absence of an exogenous metabolizing system from rat liver S9-Mix. Nitro musks revealed no genotoxicity either in the SOS chromotest with E. coli PQ37 or in the sister-chromatid exchange test with human lymphocytes.

Keywords: Nitro musks; musk xylene; musk ketone; musk ambrette; musk moskene; musk tibetene; genotoxicity; SOS chromotest; sister-chromatid exchange test; human lymphocytes

1 Introduction

Nitro musks are used as substitutes for natural musk. Chemically nitro musks are a group of synthetic dinitro- and trinitro- substituted benzene derivatives. Because of their low production costs, synthetic nitro musk compounds are of great industrial importance [1, 2, 3, 4, 5]. Musk xylene and musk ketone were detected in respectively 100 % and 80 % of 74 Japanese aquatic environment samples (freshwater fish, marine shellfish) in 1980 and 1981. The average amounts of musk xylene were 0.054 mg/kg viscera of freshwater fish, 0.016 mg/kg fish muscle and 0.003 mg/kg marine shellfish; the mean levels of musk ketone were 0.031 mg/kg viscera, 0.008 mg/kg muscle and 0.002 mg/kg shellfish [6]. LIEBL and EHRENS-TOERFER [7] identified nitro musks in human milk. The analysis of 391 milk samples from Southern Bavaria, Germany in 1991 and 1992 revealed a content of musk xylene ranging from 0.01–1.22 mg/kg fat with a mean content of 0.10 mg/kg fat. Mean contents of musk ketone and musk ambrette were 0.04 mg/kg fat. These levels are typical for various chlorinated compounds like hexachlorocyclohexanes in human fat tissue. RIMKUS and WOLF [8, 9] found similar concentrations of musk ketone-, musk xylene- and musk ambrette residues in aquatic systems, human milk and fat samples. Musk moskene and musk tibetene could not be detected.

Because of these high concentrations, the bioaccumulation and sparse toxicological data it is necessary to investigate genotoxicity. In the present study, nitro musks were tested for SOS inducing potency in the SOS chromotest with E. coli PQ37 and for sister-chromatid exchange inducing activities in human lymphocytes in vitro in the presence and absence of an exogenous metabolizing system from rat liver S9-Mix.

2 Material and Methods

Nitro musks were obtained from Promochem (Wesel, FRG; purity: musk ambrette 99 %; musk ketone 99 %; musk xylene >98 %; musk tibetene 99 %; musk moskene 99 %).

The SOS chromotest is a short-term assay for the identification of sfiA gene-inducing DNA damage caused by chemical compounds in the strain Escherichia coli PQ37 [10, 11, 12].

The procedure of the genotoxicity test that was used is described by QULLARDET and HOFNUNG [10] with modifications described in detail by Mersch-Sundermann et al. [13, 14, 15]. A sample (100 µl) of a frozen (-80 °C) E. coli PQ37 culture was thawed, suspended in 10 ml of LA medium (Difco, Nordwald, Hamburg, FRG) and incubated with agitation at 37 °C for exactly 13 hrs (overnight culture). 100 µl of this culture was added to 5 ml of fresh LA medium and incubated until it reached a photometric transmission of 40 % at 600 nm (2.0–2.5 hrs). 0.25 ml of that culture was diluted with 9.75 ml nutrient broth. Subsequently, 600
μl portions of this diluted solution were filled into a set of tubes, each containing 20 μl of the chemicals that were to be tested.

Using the procedure in the presence of an exogenous metabolic activation mixture, a modified S9 Mix containing 27 μl/assay 9000xg supernatant of Arochlor-1254 induced rat liver homogenate (Organon Technika, Eppelheim, FRG) was replaced in the nutrient broth [16].

These mixtures were incubated for 2.5 hrs at 37 °C. 310 μl from each tube were then removed and filled into new tubes, yielding two equal sets of tubes each containing a volume of 310 μl. In order to determine the β-galactosidase activity induced by DNA damaging compounds, 2.7 ml of βg-buffer and 600 μl of 0.4 % 4-nitrophenyl-β-galactopyranoside (ONPG substrate) (Boehringer, Mannheim, FRG) solution were added to each tube of one of the sets.

To determine the constitutive alkaline phosphatase activity (toxicity assay) ap-buffer replaced βg-buffer and 0.4 % 4-nitrophenyl phosphate (PNPP substrate) (Boehringer, Mannheim, FRG) solution replaced the ONPG solution.

All mixtures were incubated at 37 °C. After 25 min, the conversion of ONPG was stopped with 2 ml of 1 M sodium carbonate, and that of PNPP with 2 ml of 1.5 N sodium hydroxide. Absorption was measured at 405 nm using a reference solution with no bacteria. Positive controls, reagents, media, equipments and sources are described in MERSCH-SUNDERMANN et al. [13, 15, 17, 18].

The β-galactosidase (βg) and alkaline phosphatase (ap) activities were calculated according to the simplified method recommended by QUILLARDET and HOENING [10]: enzyme units (U) = A405 x 1000/t (A405 = optical density at 405 nm; t = substrate conversion time in minutes). The induction factor (IF) was calculated as the ratio Rx/Ro (Rx = βg/ap determined for the test chemical concentration Kx to K0; Ro = βg/ap with k=0, negative control). A compound is classified as "not genotoxic" if the induction factor remains <1.5, as "marginal" if the induction factor is between 1.5 and 2.0, and as "genotoxic" if the induction factor exceeds 2.0 and a continuous increase of the βg-activity with increasing compound concentration is found.

The sister-chromatid exchange test in vitro was performed as follows [19, 20, 21].

For the human lymphocyte in vitro sister-chromatid exchange (SCE)-test cultures were prepared from fresh blood collected aseptically by venipuncture from healthy non-smoking donors aged 25-35 yrs. Each culture consisted of 0.3 ml blood in 5.0 ml chromosome-medium (Chromosome-medium B PHA-M from Biochrom, Berlin FRG). 5-bromo-2'-deoxyuridine (Serva, Heidelberg, FRG) at a final concentration of 6 μg/ml was added to each culture.

 Cultures were treated for 24 hrs with various concentrations of each test compound in the absence of a metabolic activating system. In a second experimental setup, lymphocytes were treated in the presence of rat-liver S9-Aroclor 1254 (Organon Technika, Eppelheim, FRG) for 2 hrs (1 mg total protein content/ml culture medium). In the case of metabolic activation, rat-liver S9 was added 48 hrs after the start of culturing. Nitro musks were tested in concentrations up to a cytotoxic response in the culture determined by an absence of dividing cells. All nitro musks tested were dissolved in 50 μl DMSO, pure DMSO was used as a negative control.

In total, lymphocytes were cultivated for 72 hrs at 37 °C. 10 μg/ml colcemid was added after 70 hrs. As positive control, cyclophosphamide was used at a concentration of 0.1 μM with and without S9 Mix. The cells were harvested and slides were prepared according to the method of PERRY and WOLFF [19] for the analysis of sister-chromatid exchanges. One hundred metaphases were scored to determine the cell-proliferation [22]. Each metaphase was classified as being in the first (M1), second (M2) or third (M3) division. The proliferation index (PRI) was calculated as follows: PRI = (1 x M1 + 2 x M2 + 3 x M3)/100. Furthermore, thirty metaphases were examined for sister-chromatid exchanges for each concentration of the test-compounds and the controls.

3 Results and Discussion

To determine the DNA damaging properties of nitro musks, we investigated musk ambrette, musk ketone, musk xylene, musk tibetene and musk moskene for the induction of the SOS system in E.coli PQ37.

None of the compounds revealed genotoxic effects in the SOS chromotest in the presence or absence of an exogenous metabolizing system from rat liver (data not shown). Nitro musk compounds were tested in different concentrations until the limit of solubility of the compounds in the aqueous medium of the assays. The enzyme-activities of alkaline phosphatase and β-galactosidase showed a constant level; no bacteriotoxic effects were detected. The induction factor of the positive control 4-nitroquinoline N-oxide (in the absence of S9) was 31.43 and of benzo(a)pyrene (in the presence of S9) 5.72. These results are in accordance with those obtained by EMIG et al. [23] and MERSCH-SUNDERMANN et al. [24]. In the Salmonella/mammalian microsome test (Ames test), only musk ambrette exhibited mutagenic activity in strain TA 100 with S9 [23, 24, 25, 26]. The non-responsiveness of musk ambrette in the SOS chromotest and its positive result in the Salmonella microsome test was unexpected regarding the high concordance of the two assays in detecting genotoxicants [27, 28, 29]. Furthermore, WILD et al. [5] detected a significant effect in Drosophila with musk ambrette.

In the sister-chromatid exchange test in vitro with human lymphocytes the nitro musk compounds musk ambrette, musk ketone, musk xylene, musk tibetene and musk moskene did not increase the number of SCEs compared to non-treated lymphocytes either in the presence or in the absence of the external metabolic activating system (Table 1). Nitro musk compounds were tested in different concentrations until the limit of cytotoxicity. The SCE frequencies induced by cyclophosphamide, which served as a positive control, were 54.10 ± 14.38 in the presence of S9 and 13.94 ± 3.89 in the absence of S9.