

Preparation of Contaminant-Free 2-Hydroxyanthraquinone used in Covance Study No. 22692-0-409OECD: *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay with 2-OH-Anthraquinone

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No commercial preparation of 2-hydroxyanthraquinone (2-OH-AQ) was found that did not contain substantial contaminating material. For example, a commercially produced lot of 2-OH-AQ was purchased from Accord Chemical. Our HPLC testing program showed that that preparation of 2-OH-AQ contained significant levels of multiple contaminants including anthrone, 1-chloroanthraquinone and 2-chloroanthraquinone. The contaminant level of the 1-chloroanthraquinone alone was 0.64%. An attempt to purify the product via a re-crystallization process from ethanol and acetonitrile failed to reduce the impurities by more than 50%. It was concluded that use of a commercial source and standard purification techniques for 2-OH-AQ would not yield a product of sufficient purity to be used in the bacterial mutagenicity assays.

Accordingly, it was concluded that the 2-OH-AQ would have to be synthesized, purified, and analyzed in our laboratories. The test material was synthesized utilizing the following procedures. Amino-anthraquinone was converted to the hydroxyanthraquinones by the classical diazotization of the amine bisulfate. The diazo salts were hydrolyzed to the hydroxyanthraquinones with sulfuric acid. The purification of the 2-OH-AQ product was done by extraction of the product with dichloromethane to separate it from amine compounds. The dichloromethane extract was washed with water; and then extracted with 1N sodium hydroxide in order to separate the product from impurities such as chloroanthraquinones and nitroaromatic compounds. The alkaline extract was acidified and the product was finally re-crystallized from ethanol to yield the final product. HPLC analysis showed that the target impurities, amino and chloroanthraquinones and nitroaromatic compounds were below method detection limits of 5 ppm. The laboratory prepared and purified 2-OH-AQ was submitted as the sample to be tested in the Covance bioassay study: Covance 22692-0-409OECD: *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay with 2-OH-Anthraquinone.

Pitfalls in Analytical Methodology for Polycyclic Aromatic Hydrocarbons and Nitro-organics in 9,10-Anthraquinone: Analysis of the Anthracene Derived Anthraquinone used in the NTP Bioassay

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Anthracene derived 9, 10-anthraquinone (AQ) is obtained from a process in which anthracene is isolated from coal tar and then oxidized to form AQ (this material will be designated as AQ-OX). Consequently, numerous contaminating polycyclic aromatic hydrocarbons and nitroaromatic compounds are often present. This complex mixture presents unusually difficult challenges for purity analysis and conventional analytical methods often fail to detect impurities of significant concern. This paper describes analysis of a sample of the AQ-OX preparation that was used in a National Toxicology Program (NTP) bioassay to evaluate carcinogenic potential in mice and rats (NTP TR-494). A rigorous analytical procedure was specifically designed to quantitate impurities in AQ-OX that can be missed by conventional techniques.

GC analysis of AQ-OX can often fail to detect substantial contamination with low levels of multiple contaminants because the minimum amount of material is applied to the column to avoid overloading. Much improved analysis can be affected if the contaminants are removed and studied separately from the main material. The methods employed used both state of the art analytical techniques along with recrystallization steps to concentrate the impurities to improve the detection limits for substances in the lower concentration ranges.

A dissolution and recrystallization technique, carried out in a sealed tube near solvent boiling temperature, was used to extract the impurities from the test sample, while allowing the 9,10-anthraquinone to recrystallize. The clear supernatant solution was then analyzed using HPLC and GC techniques with several different detectors to identify and quantify the impurities. Configurations employed included HPLC/MS, GC/AP-MS, GC/MS, HPLC/UV, and HPLC/diode array. The HPLC/MS provides confirmation of the peaks observed in the routine HPLC/UV scans. The GC/AP-MS provides high sensitivity and specificity for determination of nitroaromatic compounds. The HPLC/diode array provided full spectra of the AQ and the 9-nitroanthracene. The detection limits for the sample impurities were significantly improved to values below 5 ppm for impurities such as 9-nitroanthracene, polycyclic aromatic hydrocarbons and other impurities of interest. Analysis showed that the level of contamination was 0.6%. The individual component in the highest amount was 9-nitroanthracene at levels of 0.12 - 0.14%. Other classes included polycyclic aromatic hydrocarbons at 0.05% (including anthracene, phenanthrene, and dibenzo (a,h) anthracene); and unidentified organics and nitro-organics at 0.45% (including dibenzofuran).