Fifth draft statement on the safety of Titanium Dioxide (E171) as a Food Additive- Studies used to review the toxicokinetics and absorption of the E171 form of TiO2

In this guide

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Studies used to review the toxicokinetics and absorption of the E171 form of TiO2

50. The toxicokinetics of E171 has been addressed in a few studies. The COT have assessed 9 studies in total in their review, 7 studies in mice and rats and 2 studies in humans. A summary of all the studies is provided in Annex B.

E171 studies in rats and mice

Talamini et al (2019)

51. Groups of NRF mice (n = 22/group) were treated with 5 mg/kg bw of E171 (99.3% pure anatase, 35% nanoparticles, 201.2 ± 8.5 nm) dispersed in water (no sonication or deagglomeration). The animals were treated for 3 days/week for 3 weeks receiving a total of 9 treatments in 21 days (average daily dose of 2 mg/kg bw) with E171 or water (control) which was slowly dripped with pipette in the mouths of the mice.

52. The animals were weighed at the beginning of the experiment and observed daily. On day 21, the animals were sacrificed and the lungs, liver, stomach, spleen, kidney, brain, testes and whole intestine were removed. The concentrations of titanium were determined in 4 animals.

53. No signs of general toxicity were observed. In the brain, kidney and testes, titanium levels were 0.03 µg/g, the limit of quantification (LOQ) of the analytical method for solid tissue samples. In lungs and spleen, the levels were low, with a not statistically significant, but slightly higher deposition in spleen of E171 treated animals compared to the controls. The authors reported that “Titanium concentration was one order of magnitude greater in the small intestine compared to the above tissues and distinctly higher in the stomach, large intestine and liver”. The concentrations of titanium in treated animals were: 1.07 ± 0.38 µg Ti/g tissue in the large intestine and 0.94 ± 0.57 µg Ti/g tissue in the liver. These levels were 1.8 and 3.6 times higher compared to the controls, respectively.

Comera et al (2020)

54. In the first round of experiments adult C57BL/6 mice (12–18 weeks) (4 animals/group) were treated with a single gavage dose of 40 mg/kg of E171 (> 95% anatase, 20–340 nm (transmission electron microscopy- TEM); 44.7% nanoparticles) suspended in water and sonicated or water only as a control. In the second round of experiments, 300 µg/mL of E171 was suspended in buffer and used to fill a closed mid- jejunal loop of 10cm, pre-treated with inhibitors of tight junctions, micropinocytosis, clathrin-mediated endocytosis or raft-dependent endocytosis.

55. Animals were sacrificed at 2-, 4-, 8- and 24-hours following treatment. Confocal microscopy and micro x-ray fluorescence imaging was used to analyse the existence of particles in both the first and second round of experiments, whilst ICP-MS was used to determine titanium concentration in blood and tissues (jejunum, ileum, colon). The jejunal or colonic intraluminal contents were recovered by gentle scraping.

56. In mice treated with a single dose of E171, the number of titanium dioxide reflective particles in the lumen of the small intestine was significantly increased, with analysis of the particles suggesting that no further agglomeration of titanium dioxide occurred during its transit through the intestinal tract, and that as it moved in the distal intestine, there was a decrease in its agglomeration state (as indicated by smaller particle size in the colonic versus the jejunal lumen).

57. An increase in the reflective particle content was observed in the jejunal and ileal villi, Peyer’s Patches and colon crypts. The overall particle content in jejunal villi increased from 2 hours after gavage, peaked at 4 hours, and returned to basal values at 8 hours. A statistically significant increase (p 0.001) 4 hours after

E171 administration was observed in the titanium dioxide particle density in the jejunal mucosa (increased by 3.4-fold over the controls). A lower and non-significant trend of increased particle content was also observed at 4 hours in the ileum and colon, with the values decreasing close to control levels at the time 8 hours in all three intestinal sections. In the jejunum, the reflective TiO2 particle spots displayed a mean diameter of 700 ± 59 nm (n = 70). From the content in the intestines and the weight of the mice tissues, the authors calculated that approximately 0.007% of the titanium administered was present in the entire intestine at the 4 hours timepoint. The authors concluded that titanium dioxide was absorbed predominantly in the ileum, partly in jejunum and that small amount absorbed in the colon. Based on the surface area information it was concluded that titanium dioxide is predominantly absorbed by the small intestinal villi and to a lesser extent through Peyer's patches.

Riedle et al., (2020)

58. C57BL/6 mice (6 males and 6 females per treatment group) were fed diets with food grade titanium dioxide (anatase, with a diameter of 119 nm) doses of 0, 6.25, 62.5 and 625 mg/kg of diet. These were equivalent to 0 and approximately equal to 1, 10 and 100 mg food grade TiO2/kg bw per day.

59. The animals were sacrificed at 6, 12 and 18 weeks. Animals sacrificed at 18 weeks were also used to validate that the diet permitted uptake in the intestinal lumen. The basal regions of the Peyer's patches were surveyed, and reflectance confocal microscopy was used to determine the presence of titanium dioxide.

60. Reflectant foci, indicative of titanium dioxide presence, were found at the base of the Peyer's patches at all dose groups. SEM coupled to energy-dispersive X-ray (EDX) confirmed that the tissue contained subsurface particles rich in titanium. In the low and mid dose groups, weak signals were detected in the impacted cells at the base of the Peyer's patches, whereas higher signals were observed at the highest dose group.

Bettini et al., (2017)

61. The COT noted that there was evidence of significant clearance through organs, including the liver, in the Bettini *et al*. (2017) study which examined the fate of TiO2 along the gut-liver axis in adult male rats. Firstly, rats (10 per group) were dosed daily by gavage with food-grade TiO2 (E171) or TiO2 NM-105 (Paragraphs 58 – 59) (10 mg/kg bw/day) for 7 days (the control group was given water). These animals were used for tissue imaging, flow cytometry and cytokine

assays and to carry out tissue inflammation and gut permeability measurements.

62. It was found that TiO2 particles re-agglomerated *in vivo* during transit through the gut, but not after dosing with a subsequent bolus. Following absorption, light-diffracting TiO2 particles were found along the small intestine, in the colonic (large intestine) mucosa and liver of rats dosed orally with E171 for 7 days, but not in the controls. Using NanoSIMS analyses of subcellular TiO2 distribution in the immune cells of Peyer's patches after 7 days of oral exposure, Ti was detected in the gut lumen and in the colon mucosa which corresponded to the residual dose of E171. TiO2 was also found in the liver, with the highest density found close to the portal vein sinus. There was no significant change in epithelial paracellular permeability to 51Cr-EDTA observed in the E171 group when compared with control animals. This indicates that oral exposure to E171 did not affect gut permeability *in vivo*.

Farrell and Magnuson (2017)

63. A GLP-compliant study of food-grade TiO2 (anatase) was carried out in rats and performed in accordance with the OECD TG 417 to assess the absorption, distribution and excretion routes in rats after oral exposure to TiO2. The TiO2 form of interest from this study was the anatase form (D50 = 133 – 146 nm) and was incorporated into the feed at a dose equivalent to 30 mg/kg bw/day of male and female SD rats (n = 10 per sex per group). This is based on 200 ppm and assumes daily consumption of 30 g diet per day by a 200 g rat. Rats received either TiO2 or a control diet (no TiO2 added, background concentration 7 – 9 ppm) for 7 days. Following the 7 days treatment, the TiO2 was withdrawn from diet and 3 rats per sex were sacrificed at 1, 24 and 72 hours. After the TiO2 was removed from the diet, the animals were housed individually and those allocated to the 72 hours group were housed in metabolic cages and all excreta including the cage washings were kept. The Ti content of excreta, whole blood, liver, kidneys and muscle was measured using inductively coupled plasma – atomic emission spectrometry (ICP-AES).

64. TEM micrographs indicated that the anatase TiO2 exists primarily as aggregated particles up to approximately 1µm in diameter and approximately 36% of the discrete but aggregated particles are 100 nm in diameter. However, it is not anticipated that these will de-aggregate during the manufacture of pharmaceutical or food products or in the GI tract. Dynamic light scattering technique was also used to assess the aggregation of the anatase TiO2. The median particle size (d50) was determined as 336 nm. There was no measurable quantity of TiO2 that had a particle size 100 nm.

65. No adverse effects were found during clinical observations related to the consumption of TiO2. Liver, kidney and muscle samples were analysed for Ti and 65% were below the LOD. In tissues, Ti levels were below the LOD (0.1 to 0.2 mg/kg wet weight) in most rats at most time points; of a total of 270 tissue samples (90 each from liver, kidney and muscle), 176 (65%) were below the LOD. Sporadic observations of levels above the LOD were in the range of 0.1 to 0.3 mg/kg wet weight, and occurred at similar levels and frequency in TiO2-exposed rats as those in the control group.

66. The majority of Ti was excreted in feces with concentrations in urine below the limit of quantification (LOQ; 0.04 mg/L; equivalent to 2% daily dose/L) for all samples except the 0 to 24 h urine sample for one rat (0.05 mg/L); collection of urine and feces began after withdrawal of the test diet and therefore no mass balance recovery was estimated. Whole blood Ti concentrations were also below the limit of detection (LOD) in both treatment and control groups. The authors conclude that food-grade TiO2 administered in diet is not appreciably absorbed and distributed in mammalian tissues, and there is no evidence of accumulation in liver, kidney and muscle following repeated oral exposure for 7 d.

LPT (2020)

67. “A second GLP-compliant, multi-site toxicokinetics study of 5 different grades of TiO2 was carried out in accordance with OECD 417 test guidelines and a summary report of this unpublished study was submitted to Health Canada by industry (EBRC 2022). In this study, male and female CD rats (n not stated, although the test guideline stipulates a minimum of four animals per sex per dose group) received either a vehicle control or a single dose of 1,000 mg/kg bw of TiO2 administered by oral gavage and the total Ti content of whole blood was measured for 96 h post-dosing. The relative oral bioavailability of the various grades of TiO2 was compared to a soluble Ti reference substance (Titanium(IV) bis(ammonium lactato) dihydroxide solution – 50 wt. % in H2O) that was administered orally (100 mg/kg bw) or intravenously (10 mg/kg bw). The test articles included a food-grade form of TiO2 identified as E171-E which had a median particle diameter (SD) of 99.9 ± 2.0 nm and contained approximately 50-51% of constituent particles in the nanoscale (LNE 2020). The other four particles were identified as G6-3 (a rutile TiO2-NP coated with alumina and hydrophobic organic, D50 = 9.2 ± 2.0 nm), G2-5 (uncoated anatase TiO2-NP, D50 = 5.5 ± 2.0 nm), G3-1 (uncoated pigmentary rutile TiO2, D50 = 146.9 ± 5.9 nm) and G4-19 (pigmentary rutile TiO2 coated with alumina and polyol, D50 = 177.5 ± 3.9 nm). Details of the vehicle and the dispersion protocol were not provided. Blood was

collected at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h post-dosing and Ti concentrations measured by ICP-MS/MS following microwave-assisted acid digestion of whole-blood samples using H_2SO_4. This method accounts for the bioavailability of particulate TiO_2 as well as any dissolved Ti. Blood Ti concentrations of vehicle-control treated rats were highly variable with several males considered statistical outliers and excluded from data processing. The mean blood Ti concentrations of male and female rats were below 0.2 µg Ti/g blood following oral administration of all test articles. Administration of the soluble Ti reference resulted in blood Ti concentrations up to 90 µg/g blood and 0.9 µg/g blood following i.v. and oral dosing, respectively. The highest blood Ti concentrations following oral dosing were observed in the group that received the food-grade TiO_2 test item E171-E. The resulting areas under the curve (AUC) were plotted for rats exposed to the reference substance orally or by i.v. and compared to the AUC of rats that received E171-E by oral administration. The maximum relative oral bioavailability of E171-E was determined to be 0.0013% (rounded to one significant figure for the purpose of this report). The measured blood Ti levels of the other four forms of TiO_2 were below the LOD after background correction (LOD not stated). The authors concluded that oral bioavailability of all TiO_2 grades tested was close to the LOD of the analytical system. They also stated that most reagents used in the process contain low but measurable background concentrations of Ti, which makes analysis of low levels challenging. In addition, the authors reported that the background level of blood Ti in controls rats was highly variable, especially in males, which is consistent with time zero levels measured during dietary studies.” (Health Canada, 2022).

E171 studies in humans

Pele et al., (2015)

68. The test material used was E171, anatase, d50=250nm (EFSA, 2021).

69. Eight healthy volunteers (self-reported) with normal intestinal permeability were given a permeability solution. At 7am, following an overnight fast baseline, urine samples were collected. After consumption of the solution, urine samples were collected for 5 hours.

70. Baseline blood samples were also taken at 9 am. Following that, the subjects received two tablets containing 50 mg of E171 (total dose 100 mg). Blood samples were collected at: 30 minutes, 1-, 1.5-, 2-, 3-, 6-, 8- and 10-hours post

E171 ingestion. Of the 8 volunteers, only 7 completed the study as blood could not be withdrawn from the cannula of 1 subject.

71. Dark field microscopy was used to identify titanium dioxide in the blood. Random areas were visualised and the estimation of particles within each field was based on four reflective grades: 0 (5 particles/field); 1 (5-10 particles/field); 2 (10-20 particles/field); and 3 (>20 particles/field). This analysis was only performed in 5/7 subjects due to blood clotting in two subjects. ICP-MS was used to quantify titanium in the blood for 0 - 10 hours, except in two subjects for which samples could not be collected at 8 hours (2 subjects) and 10 hours (1 subject).

72. Based on the results of the dark field microscopy, it was determined that some of the ingested titanium dioxide was absorbed directly into the blood. A significant increase in positive signals was observed from 2 hours onwards and both dark field microscopy and ICP-MS demonstrated a peak in absorption at 6 hours, reaching up to 11 ng/mL and decreasing to around 5 ng/mL by 10 hours post exposure. Only the titanium levels from 6 hours post exposure onwards were significantly different than the baseline. A positive correlation between reflective grades and total titanium levels was observed.

73. The authors hypothesised that two routes of uptake in the gut were involved: one proximal (in the duodenum/jejunum) and one distal (Peyer’s patches in ileum). This was because at two hours the uptake was visible in the dark field microscopy and the levels peaked at 6 hours as determined by ICP-MS (i.e., early absorption and late peak).

Guillard et al., (2020)

74. The test material used was: titanium dioxide particles with a mean particle size of 104.9 ± 44.9 nm and a particle size distribution ranging from 20 to 440 nm, with 55% of NPs by number.

75. Human placentae were collected at term from normal pregnancies. Transplacental passage of titanium dioxide was determined using an ex vivo placental transfusion model. The samples were analysed using ICP-MS and scanning transmission electron microscopy (STEM) coupled to EDX spectroscopy for content analysis of titanium and analysis of titanium dioxide particle deposition, respectively.

76. All placental samples (n=22) contained titanium with the total content ranging from 0.01 to 0.48 mg/kg of tissue. STEM-EDX confirmed the presence of titanium and oxygen in the particle deposits seen by TEM, as well as aluminium,

silicone, iron, zinc and tin trace elements. Most of the analysed titanium dioxide particles were below 100nm. Size particle analysis of all particles indicated that 50% were below 100nm in diameter.

77. Meconium samples were also collected from nappies. In 50% of the meconium samples (total of 18 samples), titanium was detected (0.02-1.5 mg/kg). TEM-EDX analysis confirmed the presence of titanium and oxygen elements in the particle deposits, alongside silicone, aluminium, iron and zinc. Analysis of all particles indicated a diameter of 5-194nm, with 26/33 (80%) in the nano range.

78.In the transplacental passage experiment, of the 7 ex vivo isolated perfused placentae, round shaped or small particle aggregates of titanium dioxide were observed. Titanium dioxide particles were recovered in the syncytiotrophoblast microvilli and had translocated in deeper areas of the placental chorionic mesenchyme surrounding foetal vessels. The particles had a diameter of below 250 nm, with 17 of them in the nano range.

79. The authors concluded that the results indicated the passage of titanium dioxide particles across the human placenta with potential local accumulation during pregnancy, depending on the individual. The findings of the perfused placenta experiment indicated, according to the authors, that the human placental barrier is unable to completely prevent the passage of titanium dioxide from dietary sources and protect the fetus.

80. Based on both experiments (results of perfused placenta study and the titanium levels in the placenta and meconium), the authors noted that there was a need to assess the risk of titanium dioxide nanoparticle exposure in pregnant women and warranted specific attention for oral exposure to the nanosized fraction of the E171 food additive.

Studies used to review the toxicokinetics and absorption of the nanoparticle form of TiO2

Studies in rats and mice

81. Three studies investigated the kinetics of TiO2 nanoparticles using intravenous injection (Geraets et al., 2014; Disdier et al., 2015; and Kreyling et al., 2017a), where just under 100% of the administered dose was biodistributed to the organs. However, in their gavage study Kreyling estimated the absorption from the GI tract at 0.6% of the administered dose. Therefore Kreyling et al.

(2017b) concluded that the kinetics patterns of the i.v. route were very different to those of the oral route and the “i.v. injection appears not to be an adequate surrogate for assessing the biodistribution and potential health effects occurring after oral exposure to TiO2 nanoparticles”. The focus of this assessment is TiO2 as a food additive and the route of administration is oral, therefore, these studies using i.v. administration will not be considered further but the study details are included in the Study Summaries in Annex C.

82. Of the remainder of the studies that assessed TiO2-NP treatment, none were through the diet. Seven studies dosed the animals using gavage (Kreyling et al., 2017b; Geraets et al., 2014; Ammendolia et al., 2017; Chen et al., 2020; Warheit, Boatman and Brown, 2015; Tassinari et al., 2014; and Bettini et al., 2017), the study by Hendrickson et al., 2016 used intragastric administration and the study by Hendrickson et al., 2020 used an isolated intestinal loop technique.

83. The studies that used nanoparticles (not classed as food-grade or E171 by the authors) constituted a range of sizes. The majority of the studies measured individual nanoparticles (engineered nanoparticles such as NM-101 at 5-10 nm up to about 60 nm in one dimension) and the size of agglomerates/aggregates (ranged from about 80 nm up to approximately 2 µm) predominantly using transmission electron microscopy (TEM), scanning electron microscopy (SEM) and dynamic light scattering (DLS) techniques. A few studies also assessed the percentage (by number) of the particles. For example, in Ammendolia et al. (2017) the average diameter of NPs in MilliQ water was 70 – 1,200 nm with a peak at 60 – 90 nm. There was an abundance of 11% at 76 nm and only 13% had dimensions less than 100 nm. In the study by Tassinari et al (2014) TEM showed individual NPs of 20 – 60 nm and analysis using SEM showed 87% of particles were 30 – 900 nm and 48% of those were 100 – 300 nm with an average diameter of size distribution of 284 ± 43 nm. Thirteen percent of particles had dimensions 100 nm whereas the size distribution was dominated by agglomerates up to 1.6 µm in diameter.

84. The study by Kreyling et al (2017b) which looked at the distribution of [^{48}V]TiO2NP in rats after 1 hour and up to 7 days following gavage estimated only approximately 0.6% of the dose was absorbed across the intestinal barrier in the first hour after gavage. This fraction was reduced to about 0.05% after 7 days. The rest of the material is excreted in the feces. Other studies agreed that the absorption of TiO2 across the gut epithelium was very low following dosing via gavage (oral route).

85. Kreyling et al (2017b) and others (Geraets et al., 2014; Ammendolia et al., 2017; Chen et al., 2020; Warheit, Boatman and Brown, 2015; Tassinari et al., 2014; and Bettini et al., 2017) also looked at deposition of TiO2 NPs in various tissues. The tissues in which measurable levels were detected across all studies include small intestine, liver, spleen, kidneys, lungs, heart and brain. However, the studies used different nanomaterials, different dosing duration and evaluation timepoints, and different methods of analysis.

studies in humans

Heringa et al., (2018)

86. Titanium was measured using high resolution ICP-MS in liver and spleen from 15 deceased human subjects (nine women and six men) who had donated their bodies for research and educational purposes. The LOD of the method was 10 ng/g tissue.

87. TiO2 particles were detected in 7/15 liver and 13/15 spleen samples. The number-based TiO2 particle size distributions in liver and spleen were comparable and had a size range of 85–550nm and 85–720nm, respectively. In the tissues, 24% of the TiO2 particles in the number-based size distribution was 100 nm, but this fraction may be underestimated considering that the smallest titanium dioxide particle that could be detected with the method used was 85 nm.

88. The particle mass concentration in liver ranged from 0.01 to 0.3 mg titanium/kg tissue. In the spleen, the concentration ranged from 0.01 to 0.4 mg titanium/kg tissue. The average concentration in samples where titanium could be determined was 40 ng/g in the liver and 80 ng/g in the spleen.

89. Small tissue grains of liver and spleen from two subjects were analysed using SEM-EDX to visualize the titanium dioxide particles. The observed particles were composed of titanium and oxygen and were present as an aggregate or agglomerate, consisting of smaller primary particles of 75–150 nm. Presence of titanium was also confirmed semi-quantitatively by EDX analysis in dry-ashed liver and spleen samples.

Peters et al (2020)

90. Post-mortem human liver, spleen, kidney, jejunum and ileum samples were analyzed from 15 human subjects, 7 male and 8 female, who died at the age of 64– 98 years. From these persons, written informed consent was obtained during life that allowed the use of their entire bodies for educational and research

purposes.

91. The total titanium concentration in the organs ranged from 0.01 to 2.0 mg titanium/kg tissue with an average value of 0.17 mg titanium/kg tissue and a standard deviation of 0.33 mg/kg. The authors considered that this was an indication of large differences between subjects and organs. The highest concentrations were detected in the jejunum and ileum (average of 0.34 and 0.43 mg titanium/kg respectively), followed by the kidney, spleen and liver (0.08, 0.06 and 0.03 mg titanium/kg respectively).

92. The particle sizes were measured by spICP-MS and ranged between 50 and 500 nm in the different tissues (50 nm was the lower size detection limit). The titanium dioxide particle concentrations were considered by the authors to represent about 80% of the total titanium concentration.