



INFORMATION RELEASE – 30/06/2021

Supporting information on the comparative toxicokinetics of different forms of silver (Ag) and its implications for read-across

In this paper, the European Precious Metals Federation (EPMF) provides further details supporting the information release of 15 June 2021 on new evidence on the comparative toxicokinetics of different forms of silver (Ag) and its implications for read-across. More details on the study design of the comparative toxicokinetic (TK) study commissioned by the EPMF are provided as well as the raw data and explanations supporting the conclusions of the 15 June information release. In addition, more information on the status of the study is provided.

TK study design

- The TK study¹ was an *in vivo* oral route investigation using adult CD Sprague Dawley rats. The design conformed to OECD and EU guidelines², and was conducted in compliance to Good Laboratory Practice (GLP).
- Four test items were used: two **ionic Ag salts** (silver acetate (AgAc) and silver nitrate (AgNO₃); both >99.5% pure); a well-characterised **Ag nanoparticle** reference material (AgNP; surfactant stabilized aqueous dispersion with 10% Ag; d₅₀ = 15 nm); and also a sub-micron sized powder-form (AgMP; uncoated; >99% pure; d₅₀ = ~0.3 µm; crystalline powder of highly uniform spheroidal shape) of **bulk elemental silver**³ (selected to represent a reasonable worst-case type of bulk silver in terms of expected bioavailability).
- Comprehensive enabling work was done to support the main study. The AgNP and AgMP test items were characterised to confirm suitability of the test items and to confirm stability in an appropriate aqueous-based vehicle. The AgMP test item formulated in the vehicle contained less than 0.01% of nanoparticles and ionic Ag (% of total Ag). For the AgNP suspension, the ionic Ag was ~5% of the total Ag concentration.
- **Comparative toxicokinetics** data for the various test items were obtained after both single and repeated dose administration for 28-days, including the measurement of Ag levels in blood (at different timepoints) and in tissues (at termination; in brain, bone marrow, small intestine, liver, spleen, ovaries, testes, uterus). This approach facilitated a direct quantitative comparison of parameters such as bioavailability, and the delivered dose to tissues occurring after administration of the different Ag forms.
- **Multiple dose levels per test item** were tested to account for the possibility of non-linear kinetics according to dose, whilst avoiding treatment levels expected to produce significant toxicities with a potential to perturb toxicokinetics. Dose level selection (Table 1) was based on existing *in vivo* studies (mainly for ionic silver compounds and nanosilver). No previous quantitative TK information was available for AgMP so for this test item an OECD defined Limit Dose was selected as the high-dose, with the lowest dose level set with due regard for analytical detection limits. Vehicle controls were also administered in parallel to the test items. This was the first comparative *in vivo* TK study with all 4 Ag test items. Administration was done via oral gavage. Per dose level, 4 male and 4 female rats were exposed.

¹ Silver acetate, Silver nitrate, Micron-sized Silver and Nanoparticulate Silver: A comparative toxicokinetic study in rats by single and repeat administration. Sponsor: European Precious Metals Federation (EPMF). Study identifier: 8430567. Originating institution: Covance, UK.

² OECD Guidelines for the Testing of Chemicals Section 4: OECD 417 (Toxicokinetics); and also relevant ECHA guidelines, e.g. those relating to Regulation (EC) No. 1907/2006 [REACH] and the Biocidal Products Regulation [BPR, (EU) No. 528/2012].

³ Registered under the REACH Regulation and currently placed on the EU market.



Table 1. Dose level selection for the repeated dose administration.

	silver acetate (AgAc)	silver nitrate (AgNO ₃)	silver nanoparticles (AgNP)	silver metal powder (AgMP)
Dose level 1 (mg/kg bw/d)	5	5	3.6*	36
[mg Ag/kg bw/d]	[3.25]	[3.2]	[3.6]	[36]
Dose level 2 (mg/kg bw/d)	55	55	36*	180
[mg Ag/kg bw/d]	[36]	[35]	[36]	[180]
Dose level 3 (mg/kg bw/d)	175	125	360*	1000
[mg Ag/kg bw/d]	[114]	[80]	[360]	[1000]

* expressed as Ag equivalent value

- Total Ag concentrations were determined in whole blood and tissues by ICP-MS after microwave digestion with nitric acid and hydrochloric acid. This method was successfully validated with a lower limit of quantification (LLOQ) of 10 ng/mL for silver in rat blood⁴ and 30 ng/g for silver in rat tissue⁵.

More information on TK study design is provided in Annex I.

TK study results

Systemic exposure / Ag in blood

- Figure 1 and Table 2 show the extent of systemic exposure as area under the concentration time curve (AUC) values after repeated dose administration of silver acetate (AgAc), silver nitrate (AgNO₃), silver nanoparticles (AgNP) and bulk elemental silver (AgMP) for 28 days.**

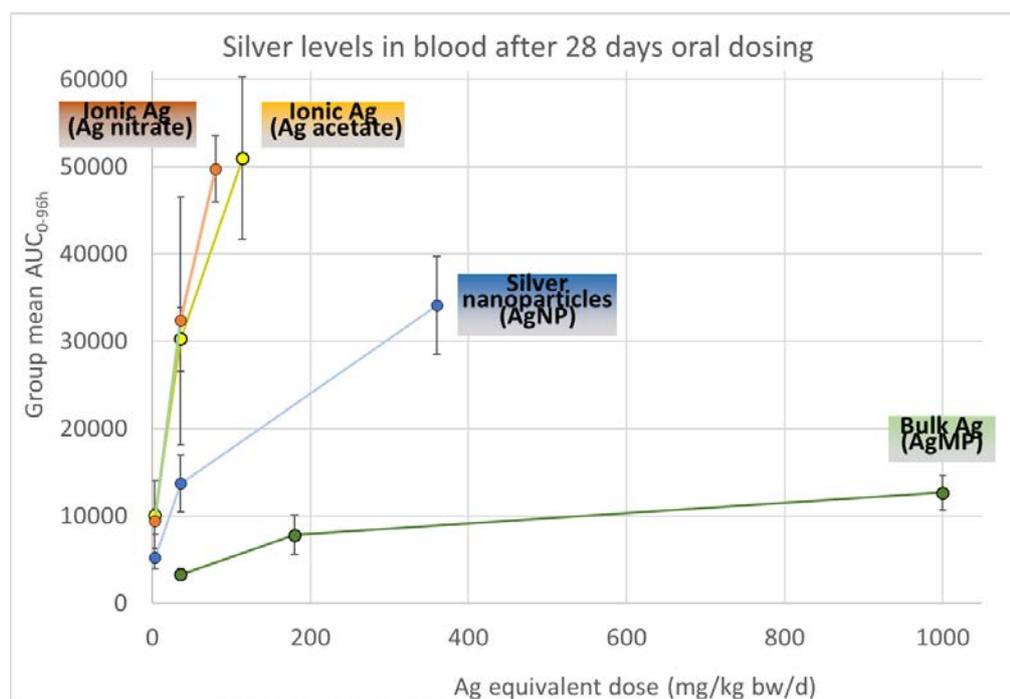


Figure 1. Marked differences exist in the extent of absorption observed for ionic Ag (tested as Ag acetate and Ag nitrate) versus that of bulk Ag (sub-micron Ag metal powder, 'AgMP').

Results are for female animals receiving the test items (very similar findings were also obtained in the case of males).

Data are expressed as Ag equivalent dose. Standard deviations are added.

⁴ Method validation study report – Validation of an ICP-MS assay for the quantitation of silver in rat blood. Study Code: 192194C. Sponsor: European Precious Metals Federation (EPMF). Originating institution: ARC Trinova Ltd (Arcinova), UK.

⁵ Method validation study report – Validation of an ICP-MS assay for the quantitation of silver in rat tissue digest. Study Code: 192194B. Sponsor: European Precious Metals Federation (EPMF). Originating institution: ARC Trinova Ltd (Arcinova), UK.



Table 2. AUC values (d.28) after repeated oral dose administration of: (a) silver acetate; (b) silver nitrate; (c) nanoparticulate silver; or (d) bulk elemental silver

(a) Silver acetate (AgAc)

Dose level (mg/kg Test Item bw/d)	Mean AUC _{0-96h} ng.h/mL		Mean AUC / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	NR	NR (n=3)	--	--
5	6148 ± 1106	10110 ± 3876	1891	3111
55	23810 ± 2132	30235 ± 3648	666	846
175	43044 ± 6159 (n=3)	50976 ± 9346 (n=3)	378	448

AUC_{0-96h} results are expressed as the sub-group mean ± SD.
Sub-group size: n=4 animals, except where otherwise indicated.

(b) Silver nitrate (AgNO₃)

Dose level (mg/kg Test Item bw/d)	Mean AUC _{0-96h} ng.h/mL		Mean AUC / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	NR	NR (n=3)	--	--
5	5963 ± 866	9346 ± 1505	1878	2944
55	22609 ± 4240	32334 ± 14206 (n=3)	647	926
125	39168 ± 10095	49751 ± 3783	493	627

AUC_{0-96h} results are sub-group expressed as the mean ± SD.
Sub-group size: n=4 animals, except where otherwise indicated.

(c) Nanoparticulate silver (AgNP)

Dose level (mg/kg Test Item bw/d)	Mean AUC _{0-96h} ng.h/mL		Mean AUC / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	NR	NR	--	--
3.6	6307 ± 569	5141 ± 1177	1752	1428
36	9762 ± 1703	13673 ± 3244	271	380
360	19011 ± 3671	34107 ± 5611	53	95

AUC_{0-96h} results are expressed as the sub-group mean ± SD.
Sub-group size: n=4 animals.

(d) Bulk elemental silver (AgMP)

Dose level (mg/kg Test Item bw/d)	Mean AUC _{0-96h} ng.h/mL		Mean AUC / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	NR	NR	--	--
36	2877 ± 606	3276 ± 676	80	91
180	5497 ± 715	7811 ± 2229	31	43
1000	8072 ± 1997	12668 ± 2019	8	13

AUC_{0-96h} results are sub-group expressed as the mean ± SD.
Sub-group size: n=4 animals.

- Comparisons via normalised administered doses (i.e. Ag equivalent dose) can be made between Ag acetate and bulk Ag (AgMP). Based on these matched dose assessments, the extent of systemic exposure was about **10 to 30-fold lower in the case of bulk Ag versus a reference ionic Ag salt**.
- Unlike the situation with ionic Ag, the degree of uptake of bulk Ag was not linear when the amount of administered bulk Ag was increased up to a limit dose. Instead, there was evidence of absorption plateauing (Figure 1).
- Table 3 shows the blood Ag concentrations at predicted steady state (day 15) after repeated dose administration of the different Ag test items. The maximum observed concentration occurs between 3-9h post-dosing for repeat dose groups, hence the 6h values for the respective test substances are shown as an indicator of peak exposure. Several previous studies reported in the literature provide information on the attainment of steady state conditions following repeated oral dosing of silver substances (both ionic silver and several types of Ag nanoparticles). For instance, work by van der Zande et al. (2012)⁶ on silver nitrate and AgNP (small capped and uncapped NP types). The serial Ag in

⁶ van der Zande et al. (2012) Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano. 6: 7427–7442.



blood measurements performed in that investigation demonstrated that steady state conditions were evident after 14 days of dosing. A similar temporal estimate of time to steady state is also supported by extrapolations made using elimination half-life data for various silver reference substances. In accordance with this, the study design incorporated an interim blood sampling occasion on day 15.

Table 3. Blood Ag concentrations at day 15 after repeated oral dose administration of different Ag test items (a = silver acetate; b = silver nitrate; c = nanoparticulate silver; d = bulk elemental silver).

(a) Silver acetate (AgAc)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean [Ag] _{6h} / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ (n=3)	--	--
5	113 ± 15	204 ± 26	34.8	62.8
55	317 ± 93	423 ± 72	8.9	11.8
175	652 ± 171 (n=3)	918 ± 103 (n=3)	5.7	8.1

Results are expressed as the sub-group mean ± SD. Unless otherwise stated, samples were obtained from 4 animals per sub-group.

(b) Silver nitrate (AgNO₃)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean [Ag] _{6h} / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ (n=3)	--	--
5	112 ± 12	170 ± 42	35.3	53.5
55	291 ± 28	377 ± 48 (n=3)	8.3	10.8
125	539 ± 113	636 ± 28	6.8	8.0

Results are expressed as the sub-group mean ± SD. Unless otherwise stated, samples were obtained from 4 animals per sub-group.

(c) Nanoparticulate silver (AgNP)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean [Ag] _{6h} / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ	--	--
3.6	117 ± 23	125 ± 52	32.5	34.8
36	174 ± 32	288 ± 36	4.8	8.0
360	263 ± 35	519 ± 101	0.73	1.44

Results are expressed as the sub-group mean ± SD. Samples were obtained from 4 animals per sub-group.

(d) Bulk elemental silver (AgMP)

Dose level (mg/kg Test Item bw/d)	Mean 6h blood [Ag] ng/mL		Mean [Ag] _{6h} / Ag Equiv. Dose ng/mL	
	♂	♀	♂	♀
Control	<LLOQ	<LLOQ	--	--
36	57 ± 17	55 ± 6	1.6	1.5
180	127 ± 25	190 ± 26	0.71	1.05
1000	154 ± 19	251 ± 71	0.15	0.25

Results are expressed as the sub-group mean ± SD. Samples were obtained from 4 animals per sub-group.

- These data demonstrate that **oral dosing of AgMP leads to appreciably lower Ag amplitude of systemic exposure** in comparison to ionic Ag forms, while AgNP exhibits an intermediate systemic exposure profile. AgAc and AgNO₃ exhibit very similar systemic exposure profiles. As an illustration of this point, Figure 2 shows the differentials in amplitude of exposure between ionic Ag forms (AgAc/AgNO₃) versus bulk Ag (AgMP).

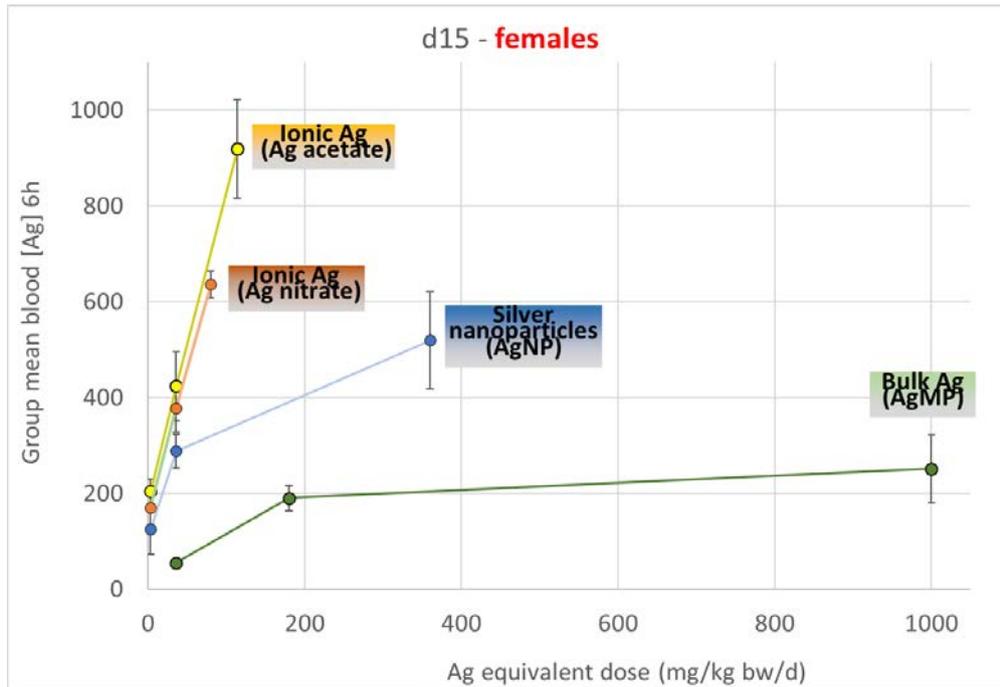


Figure 2. Ag systemic exposure after repeat oral dosing of different Ag forms.
Results are for female animals receiving the test items (very similar findings were also obtained in the case of males).

Tissue exposure / Ag distribution

Table 4 shows the Ag tissue levels / distribution after repeated dose administration of the different Ag test items.

Table 4. Terminal Ag tissue levels following repeated oral dose administration for 28 days of: (a) silver acetate; (b) silver nitrate; (c) nanoparticulate silver; or (d) bulk elemental silver

(a) Silver acetate (AgAc)

Dose level mg AgAc/kg bw/d [mg Ag/kg bw/d]	Group mean Ag in tissue concentration (ng/g tissue)								
	Spleen		Bone Marrow		Brain		Ovary	Uterus	Testis
	♂	♀	♂	♀	♂	♀			
Control	--	--	--	--	--	--	--	--	--
5 [3.25]	283 ± 71	990 ± 455	62 ± 13	126 ± 110	142 ± 36	169 ± 21	2197 ± 1075	188 ± 121	167 ± 76
55 [36]	38656 ± 11039	60783 ± 10360	3500 ± 1539	4501 ± 1421	637 ± 57	805 ± 36	24262 ± 2613	8004 ± 3300	1508 ± 215
175 [114]	96379 ± 55201 (n=3)	141560 ± 93525 (n=3)	21373 ± 15435 (n=3)	46761 ± 19839 (n=3)	1458 ± 33 (n=3)	1455 ± 114 (n=3)	39668 ± 19807 (n=3)	11094 ± 2319 (n=3)	1531 ± 236 (n=3)



(b) Silver nitrate (AgNO₃)

Dose level mg AgAc/kg bw/d [mg Ag/kg bw/d]	Group mean Ag in tissue concentration (ng/g tissue)								
	Spleen		Bone Marrow		Brain		Ovary	Uterus	Testis
	♂	♀	♂	♀	♂	♀			
Control	--	--	--	--	--	--	--	--	--
5 [3.18]	346 ± 226	2728 ± 2542	94 ± 28 (n=3)	328 ± 236	124 ± 27	206 ± 63	2518 ± 1638	313 ± 319	396 ± 171
55 [35]	40159 ± 12214	54444 ± 964 (n=3)	4102 ± 963	3901 ± 1281 (n=3)	641 ± 151	624 ± 71 (n=3)	27543 ± 1949 (n=3)	13707 ± 2395 (n=3)	1237 ± 95
125 [79]	48660 ± 3841	70183 ± 8565	15126 ± 6055	20047 ± 5178	1191 ± 181	1043 ± 88	31533 ± 1020	24740 ± 9028	1286 ± 428

(c) Nanoparticulate silver (AgNP)

Dose level mg Ag/kg bw/d	Group mean Ag in tissue concentration (ng/g tissue)								
	Spleen		Bone Marrow		Brain		Ovary	Uterus	Testis
	♂	♀	♂	♀	♂	♀			
Control	--	--	--	--	--	--	--	--	--
3.6	191 ± 83	99 ± 93	59 ± 26	40 [§]	124 ± 22	91 ± 11	368 ± 376	40 [§]	179 ± 117
36	2390 ± 1287	3522 ± 2751	335 ± 83	296 ± 192	261 ± 27	291 ± 62	7201 ± 4884	574 ± 412	570 ± 34
360	14171 ± 2803	51630 ± 13644	1580 ± 730	4921 ± 1722	568 ± 150	875 ± 119	24285 ± 6571	11235 ± 5279	1053 ± 132

(d) Bulk elemental silver (AgMP)

Dose level mg Ag/kg bw/d	Group mean Ag in tissue concentration (ng/g tissue)								
	Spleen		Bone Marrow		Brain		Ovary	Uterus	Testis
	♂	♀	♂	♀	♂	♀			
Control	--	--	--	--	--	--	--	--	--
36	89 [‡]	43 [‡]	<LLOQ	<LLOQ	58 ± 10	56 ± 14	<LLOQ	<LLOQ	<LLOQ
180	218 ± 103	276 ± 85	47 ± 7	65 ± 12	119 ± 19	128 ± 27	525 ± 276	46 ± 14	160 ± 42
1000	473 ± 258	1378 ± 328	130 ± 11	165 ± 55	171 ± 16	223 ± 29	3645 ± 2165	236 ± 127	332 ± 100

Results are expressed as means ± SD.

Unless otherwise stated, samples were obtained from 4 animals per sub-group.

LLOQ = lower limit of quantification.

[‡] only two samples in the group had measurable levels.

[§] includes interpolated results based on LLOQ



- The data demonstrate that **levels of Ag distributed into tissues and organs are considerably lower in the case of bulk elemental Ag** than an ionic Ag compound. This links to predictions that bulk silver represents a correspondingly lower health hazard, i.e., is less likely to cause toxic effects.
- The silver nanoparticle reference material (AgNP) exhibits an intermediate TK profile with achieved tissue levels more closely resembling those of the ionic Ag test items rather than those evident for bulk Ag (AgMP).
- It should be noted that Ag levels in small intestine and liver are not presented as these analytical measurements were unreliable due to a cross-contamination event. This part of the TK study is currently being repeated and results are expected in November 2021.

Conclusions

It is commonly considered that the systemic toxicity of inorganic silver substances is driven by the silver ion (Ag⁺) as the primary species relevant to tissue exposure, and hence hazard assessment. The EPMF comparative TK study is a high quality GLP investigation and the first which permits a direct comparison of ionic Ag, nanosilver and bulk silver (tested as sub-micron sized powder-form) – in terms of **relative oral uptake and tissue distribution of Ag⁺**.

- The study outcomes confirm that oral intake of **bulk elemental silver results in markedly lower systemic exposure than seen for more bioavailable forms**.
- This conclusion from the experimental model is expected to be **relevant to humans**.
- The study findings strongly suggest that the direct read-across of mammalian toxicity datasets for simple ionic silver salts (like silver acetate) and nanosilver to bulk silver is **not supported** (from a scientifically valid toxicokinetic perspective).
- Hence, we ask that certain **read-across assumptions** within the existing CLH proposal for silver⁷ are **reconsidered, particularly in respect of the assessment of bulk silver forms**.
- It should be emphasised that the bulk elemental silver tested in the EPMF comparative TK study had a median particle size of ca. 0.3 µm and thus represent a worst-case type of bulk silver in terms of expected bioavailability. The differential between bulk elemental silver and more bioavailable silver forms is thus already evident at low particle sizes of bulk silver.
- With due regard of this newly available scientific information, the European Chemicals Agency (ECHA) Read-Across Assessment Framework (RAAF)⁸ should be appropriately applied to better structure the scientific evaluation of grouping and read-across for elemental silver forms. Metals are within scope of this schema, and RAAF acknowledges that differences in relative bioavailability of metal species represent a basis for differentiation in read-across/grouping.

⁷ Proposal for Harmonised Classification and Labelling under Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2. Substance Name: Silver (CAS Number: 7440-22-4). Prepared by Swedish Chemicals Agency (KEMI), Version number: 3. Dated: September 2020. Released by ECHA for public consultation on 19 October 2020.

⁸ The European Chemicals Agency (ECHA) has codified a systematic and consistent approach to assessing such read-across situations, viz. their Read-Across Assessment Framework (RAAF). ECHA-17-R-01-EN. ISBN: 978-92-9495-758-0. March, 2017.

This Information Release has been prepared with the assistance of Raffray Biosciences Ltd.



Annex I: Material and Method

1. Animal information

CD Sprague Dawley rats, accepted by regulatory agencies, background data available.

Animals' information	
Species	Rat
Strain	CrI:CD IGS (SD).
Sex	Male and female
Age range ordered	8 - 10 weeks old at start of treatment (49-55 days old on arrival)
Weight range ordered	A minimum of 170 g for females and 200 g for males.
Supplier	Charles River UK
Acclimatization duration	At least 5 days before commencement of treatment
Diet supply	SDS VRF-1 – pelleted diet – non restricted (diet contains no added antibiotic or other chemotherapeutic or prophylactic agent)
Water supply	Potable water from public supply – non restricted via polycarbonate bottles with sipper tubes
Allocation to treatment group method	Random
Cage distribution	Within each group, cages will be blocked together (by sex). The distribution of the groups within and between racks and the position of the racks within the room will be determined to equalize environmental influences across the study while minimizing the opportunity for inter-group contamination
Identification	Unique number for each animal by microchip

2. Test Item and Supporting information

Silver Acetate (AgAc)	
CAS Number	563-63-3
Alternative identity	AgAc; AG(I) Acetate T2 HSTDP; Silver(I) Acetate
Molar mass	166.91 g/mol, (of which 107.87 g/mol Ag)
Solubility	Aqueous (room temperature): 1.02g/100mL or 10.2 mg/mL
Storage conditions	Room temperature; protected from light
Purity	>99.5%
Appearance	Colourless to pale-white crystalline powder

Silver Nitrate (AgNO ₃)	
CAS Number	7761-88-8
Alternative identity	AgNO ₃ ; Silver(I) Nitrate
Molar mass	169.87 g/mol, (of which 107.87 g/mol Ag)
Solubility	Aqueous (room temperature): 256 g/100mL or 2560 mg/mL
Storage conditions	Room temperature; protected from light
Purity	99.97% (assay)
pH	4.6 as 10% solution
Appearance	White crystalline powder



Micro-sized silver (Ag MP)

CAS Number	7440-22-4
Alternative identity	Ag MP; Bulk Silver; Bulk Ag; Micron-size silver particulate (Ag MP); S11000-25 Ag Powder; Silver powder and silver flake
Molar mass	107.87 g/mol
Composition	Uncoated silver particulate (crystalline Ag powder of highly uniform spheroidal shape)
Particle size	~0.4 µm
Particle size range	PSD: d90; 0.53 µm / d50; 0.35 µm / d10; 0.24 µm
Density (tapped)	3.5 kg/dm ³
Storage conditions	Room temperature; protected from light
Purity	>99%
Appearance	Silver/grey powder

Nanoparticle Silver (Ag NP)

CAS Number	7440-22-4
Alternative identity	Ag NP ; Agpure® W10
Molar mass	107.87 g/mol
Composition	Surfactant stabilized aqueous dispersion of nanoparticles containing 4% (w/w) polyoxyethylene glycerol (25) trioleate [TAGAT™ TO] and 4% (w/w) polyoxyethylene (20) sorbitan mono-laurate [Tween 20™] together with 7.5% (w/w) ammonium nitrate
Particle size	15 nm (mean)
Particle size range	PSD: d99 20 nm
pH (dispersion)	7.0 – 9.0
Density	1.1 kg/dm ³
Storage conditions	Room temperature; protected from light
Purity (silver concentration)	Specification is for an elemental silver concentration of 10.0 ± 0.50 % wt (100 mg/mL). Supplied as a suspension in 4% polyoxyethylene (PEG 25) glycerol trioleate and 4% Tween 20
Appearance	Dark orange liquid



3. Treatment Groups

4 weeks (28 d) Repeated Dose (Oral Gavage)														
	Vehicle	Ag MP			Ag NP			Vehicle	AgAc			AgNO ₃		
Dose level (mg/kg/day)	1% w/v aqueous methyl cellulose	36	180	1000	3.6*	36*	360*	water	5	55	175	5	55	125
Target Concentration (mg/mL)		1.8	9	50	0.18*	1.8*	18*		0.25	2.75	8.75	0.25	2.75	6.25
Volume (mL/kg/day)	20	20			20			20	20			20		
Animal No./sex/dose	4M+4F	4M+4F			4M+4F			4M+4F	4M+4F			4M+4F		

MP – micron size particulate NP – nanoparticulate * Silver equivalent value

Correction factor:

- Except for AgNP formulations, correction factors for content or purity was required.
- For AgNP, the Test Item as supplied, is a pre-formulated liquid preparation of NP silver at a concentration of 100 mg/mL (10% w/v), and thus a correction factor of 10 was used to ensure the amount of Test Item aliquoted for formulation is adjusted to provide the required concentration of silver in the final volume.

4. Test Item preparation

4.1. Vehicle trials

Suitable vehicles for formulation of each Test Item and the routes of administration was assessed prior to the start of the study. The pH of test formulations was measured.

Obvious changes of state such as agglomeration behaviour or visible change of colour was assessed. Any such undertakings was recorded in the raw data..

In respect of the Ag MP and Ag NP Test Items, multi-parameter characterization investigations was undertaken by the Sponsor using vehicles equivalent to those selected for this study.

4.2. Vehicles (AgMP and AgNP):

- Oral doses - 1% w/v aqueous methylcellulose (400 cps)
- Prepare each vehicle in advance following standard methods and store refrigerated until required for use, then bring to room temperature prior to use.

4.3. Vehicles (AgNO₃ and AgAc)

- Water for Injection (for oral doses)
- Formulation containers:
 - Polypropylene vessels and containers will be used throughout preparation and storage. Vessels should be pre-cleaned with a dilute nitric acid solution (11.5 mL of



concentrated HNO₃ per litre of pure water) and rinsed thoroughly with ultrapure water.

- Prepared final formulations should be protected from light, e.g. via use of dark-coloured vessels and/or non-transparent foil wrapping

4.4. Method of preparation

- **Formulation procedure:**
 - Chloride and phosphate containing vehicles and reagents was avoided due to the potential for silver precipitation effects.
 - Dissolved silver and silver particles adsorb onto material surfaces including glass and metals, and as such use of these was avoided.
 - Formulations was protected from light during preparation (use of yellow-light instead).
 - The formulation procedures is documented in the study data and will be included in the final report.
- **Method of Preparation for Silver salt (AgAc and AgNO₃) formulation; Oral gavage:**
 - The pH of the final formulations was measured and recorded in the data
 - A series of solutions at the required concentrations was prepared by dilution of individual weighing.
 - The method of preparation:
 - The required amount of test item was weighed out, (Ag Acetate was ground in a pestle and mortar) and then added to ca 50% of the final volume of vehicle and magnetically stirred to mix/disperse. The 'weighing' vessel was rinsed, and the rinsing is added to the container before bringing to volume with the vehicle. The mixture was then transferred to a magnetic stirrer and stirred for a minimum of 20 minutes, recording the start and finish times in the raw data. Formulation analysis sampling will occur whilst under constant stirring, prior to dispensing and aliquoting into suitable containers for dose administration (stirrer bars will be included).
- **Method of Preparation for Ag NP and Ag MP (oral gavage formulation):**
 - The pH of the final formulations should be measured and recorded in the data.
 - A series of formulations at the required concentrations will be prepared by dilution of individual weighing (or in the case of AgNP, individual volumes) of the Test Item and dispensed in polypropylene screw top jars or similar
 - Method of preparation:
 - 50% of the final volume of vehicle will be added to an appropriate container. The required amount of Test Item will be weighed and added to the vehicle whilst stirring at an appropriate speed to obtain a vortex that is half the depth of the suspension. The 'weighing' container will be rinsed several times using 20% final volume of vehicle. After addition of the rinsing, the suspension will be stirred for 60 seconds and then sonicated for approximately 30 minutes. This stir and sonicate step will then be repeated, after which the remaining 30% final volume of vehicle will be added and stirring continued at an appropriate speed to obtain a vortex that is half the depth of the final suspension for at least 60 minutes.
 - The suspensions will be sampled for formulation analysis (whilst under constant stirring, according to the previously described technique) prior to dispensing and aliquoting into suitable containers for dose administration. (stirrer bars will be included).



4.5. Frequency of preparation:

- Repeat dose formulations - Weekly and split into daily aliquots. Could be prepared in advance of the first day of dosing.
- The AgNP and AgMP formulations required additional conditions. The oral gavage formulations prepared no more than 4 days prior to first use of each preparation.
- The suitability and homogeneity of formulations were confirmed as part of studies, undertaken separately by third parties designated by the Sponsor, and by the Principal Investigator for dose formulation analysis.

4.6. Storage of formulation:

- Silver suspensions (AgMP & AgNP) – refrigerated (2 to 8°C), protected from light.
- Silver solutions (AgNO₃ and AgAc) – ambient temperature (15 to 25°C), protected from light.
- Solutions of AgAc should not be subjected to refrigerated temperatures due to the risk of precipitation.
- These conditions are based on the outcomes of characterization investigations undertaken separately by 3rd parties on behalf of the Sponsor.



5. Method administration Oral administration

Route	Oral gavage.
Handling	Solution formulations (AgAc & AgNO ₃) - As per SOP Suspension formulations (AgMP & AgNP) – It imperative that these formulations are magnetically stirred at an appropriate speed to obtain a vortex that is half the depth of the final suspension for a minimum of 20 minutes prior to administration and then stirred continuously throughout the dosing procedure. Formulations should be protected from light during dosing and storage.
Treated at	Constant doses in mg/kg/day
Volume dose	20 mL/kg body weight.
Individual dose volume	Calculated from the most recently recorded scheduled body weight.
Controls	Vehicle at the same volume dose as treated groups.
Frequency	Repeat dose groups: Once daily at approximately the same time each day.
Sequence	By phase and group.
Dosing procedure	To aid animal compliance and lubrication during the dosing procedure the use of vehicle (that used in dosing) will be employed as a dip for the exterior of the catheter. alternative dips may also be considered where explicitly authorized by the SD and Sponsor and documented in the study data.
Storage of formulation	AgAc & AgNO ₃ formulations - Room temperature (15 to 25°C). AgMP & AgNP formulations - Refrigerated (2-8°C).
Formulation	A record of the usage of formulation will be maintained based on weights. This balance is compared with the expected usage as a check of correct administration.
Residues (Repeat dose phases)	It has been requested that residues from the repeat dose phases be retained frozen as contingency (-20°C or below) until further notice.

6. Duration of Treatment

For welfare, observational and logistical reasons, test item administration was staged across several dose phases. Each repeat dose phase will comprise 28 days of dosing following by up to 96 hours of sampling prior to termination for tissue sampling.

The observation period may be extended, to incorporate any additional observations considered necessary; documented in an amendment to study plan.

Throughout any additional study periods and during the necropsy periods the serial observations will be recorded at appropriate intervals. Data for any additional complete weeks before commencement of necropsies will be included in the final report.



7. Rationale for Dose Level Selection and Route

AS APPLICABLE, THE SELECTED DOSE LEVELS ARE SCALED FOR SILVER EQUIVALENT CONTENT IN RESPECT OF SILVER SALTS AND ELEMENTAL SILVER FORMS.

The dose levels for silver acetate (AgAc) have been chosen to support a program of reproductive toxicology studies on this compound. Based on existing toxicity data for AgAc, from repeated administration studies in rodents the dose levels are expected to be well tolerated.

The dose levels selected in the case of silver nitrate (AgNO₃) extend existing but limited toxicokinetic data for this compound. A previous 28-day toxicity study demonstrated that up to 100 mg/kg bw/d of AgNO₃ administered to rats via oral gavage produced minimal toxicity. The high-dose treatment level has been set with due regard for the local tissue irritancy profile of AgNO₃.

No reliable toxicokinetic information is currently available for micron-size bulk silver (Ag MP) and this study is intended to close that data gap. Whilst very limited general toxicity data are currently available for Ag MP via the oral route, bulk silver forms are not known to produce significant systemic effects or to cause local tissue reactivity. Furthermore, *in-chemico* studies suggest that the bioavailability of this silver form will be more limited than the other Test Items. Hence it is expected that a dose level of 1000 mg/kg bw/d via the oral route, which corresponds to an OECD defined Limit Dose, will not produce any appreciable toxicities.

The dose levels selected in the case of nanoparticulate silver (Ag NP) extend existing but limited toxicokinetic information which is available for this form of silver. Previous repeat dose studies in rats via the oral route for durations up to 13 weeks have shown that no marked systemic toxicity or local tissue reactivity has occurred with Ag NP dose levels of up to 500 mg/kg bw/d. Therefore, it is expected that the high-dose group level selected for this study (360 mg/kg bw/d) should be well tolerated.

8. Regulatory Testing Guidelines

There are no specific regulations applicable to the study, however consideration was given to the following during the design of the study:

- COUNCIL REGULATION (EC) No 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
- OECD Guidelines for the Testing of Chemicals Section 4: OECD 417; Toxicokinetics (22 July 2010).

Good Laboratory Practice:

With the exceptions stated below, the study including any phases conducted by the Principal Investigator, will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

- The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994)
- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17
- EC Commission Directive 2004/10/EC

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Each Principal Investigator will be responsible for compliance with OECD GLP and their national GLP regulations.



No claim of GLP compliance will be made for the vehicle trials as part of this study, where undertaken and due to the nature of the Test Item and analyte, stability of the test formulation under conditions of storage cannot be directly assessed as part of this study. Additionally, no claim of compliance is made for test item manufacture, test item expiry dates (where not available or applicable), or for transportation of test items prior to receipt at the test facility.

Animal Welfare

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (the Act).

The number of animals used will be the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.