Additional information report

For a Substance under Harmonised Classification and Labelling Process

Substance Name: Silver

EC Number: 231-131-3

CAS Number: 7440-22-4

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Date: 29 April 2022

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1 INTRODUCTION

During the CLH process of silver, ECHA received a copy of the following newly performed studies:

- Silver acetate, Silver nitrate, Micron-sized Silver and Nanoparticulate Silver: A comparative toxicokinetic study in rats by single and repeat administration (Melvin et al., 2021)
- Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Repeat administration (Charlton et al., 2021)

To assure an efficient and transparent assessment of the new information provided by the data submitter and giving parties concerned an opportunity to comment, it is the responsibility of the data submitter to elaborate non-confidential detailed study summaries and explain the relevance of the provided information for the classification at issue. To facilitate the task of the data submitter, ECHA has compiled a format for Additional information report.

Every study should be presented individually. The relevance of each study for the classification at issue should be clearly presented. A non-confidential detailed study summary and the results of the study should be reported transparently and objectively as in the original data source without any interpretations by the data submitter. A detailed study summary includes test type (e.g. test guideline followed and deviations from the guideline, the GLP and quality assurance status of the study, identity of the test substance (including degree of purity), information about the test subjects, route of administration, mode of exposure and numerical results). If read-across to structurally or mechanistically similar substances is used a justification for using data from a different substance should be provided and, if known, calculations to convert dose/concentration levels from the test substance to the substance for which CLH is proposed should be presented.

2 TOXICOKINETIC STUDIES

The EPMF *in-vivo* comparative TK study (according to OECD TG 417) is a high quality GLP investigation and the first which permits a direct comparison of soluble silver compounds, nanosilver and silver powder (tested as a sub-micron sized powder-form) – in terms of relative oral uptake and tissue distribution of silver. The study consists of **two study reports** and the findings obtained for the different forms of silver across the two study segments can be compared as both segments were performed according to an exactly equivalent experimental design, under the same conditions and at the same contract laboratories (for both the in-life and bioanalytics phases). Comparison of AUC profiles obtained after repeated oral dosing confirm that oral intake of silver powder results in markedly lower systemic exposure than seen for soluble silver compounds and nanosilver. When looking at differentials in silver in tissue concentrations following repeated dosing, for the majority of tissues, exposure to soluble silver compounds leads to tissue Ag levels which are 2 to 3 orders of magnitude higher than those evident for the silver powder. The tissues for which the difference is less than 2 to 3 orders of magnitude, are minor sites of distribution. The study shows that the testis and brain are minor sites of distribution. In contrast, the ovary has been shown to be an important site of distribution. However, ancillary investigations strongly suggest silver is present in the ovary as chemically stable silver sulphide/selenide complexes with very low local bioavailability. The study findings strongly suggest that the direct read-across of mammalian toxicity datasets for soluble silver compounds (like silver acetate) and nanosilver to silver powder is not supported (from a scientifically valid toxicokinetic perspective).

Study references:

1. Melvin P et al. (2021) Silver Acetate, Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Single and Repeat Administration. Labcorp Study Number: CC71MP.

2. Charlton A et al. (2021) Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Repeat Administration. Labcorp Study Number: 8469368.

3 RELEVANCE OF STUDIES

Studies are relevant for the classification of Ag metal for systemic and repeated dose toxicity because they measured the difference in release and distribution of ionic silver [Ag^+] (toxicophore) between silver micron-sized powder (AgMP), nanosilver (AgNP), silver nitrate ($AgNO_3$) and silver acetate (AgAc). In particular, considerably lower systemic and tissue-level exposure were evident for AgMP versus either ionic (AgAc and $AgNO_3$) or nanosilver (AgNP) references. These *in-vivo* comparative toxicokinetic studies thus do not support a direct read-across but rather a weight of evidence approach of recent studies performed with AgAc (i.e., the 90-day study and the Extended One-Generation Reproductive Toxicity Study (EOGRTS) and its preliminary study) to silver (massive and

powder), with the latter displaying a quantitatively very different toxicokinetic character and a much lower release rate of Ag^+ than silver acetate or silver nitrate.

4 Detailed study summary and results of Melvin et al., 2021 study

Silver Acetate, Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Single and Repeat Administration. Labcorp Study Number: CC71MP

Information substance identity

Test item: Micron-sized Silver
Test item identify: Referenced as AgMP throughout the study report
Alternative names: AgMP; Bulk Silver; Bulk Ag; Micron-sized silver particulate; Silver powder and silver flake
CAS number 7440-22-4
Intended use: Chemical feed stock
Appearance Silver/grey powder
Composition: Uncoated silver particulate (crystalline Ag powder of highly uniform spheroidal shape)
Particulate size ~ 0.4 µm
Particulate size range: PSD: d90; 0.53 µm / d50; 0.35 µm / d10; 0.24 µm
Density (tapped): 3.5 kg/dm^3
Storage condition: Room temperature: protected from light
Expiry date: 29 June 2021
Purity: > 99%

Test item: Nanoparticulate Silver
Test item identity: Referenced as AgNP throughout the study report
Alternative names: AgNP, nanoAg, nanosilver
CAS number: 7440-22-4
Intended use: Chemical feed stock
Appearance: Dark orange liquid
Composition: Surfactant stabilized aqueous dispersion of nanoparticles containing 4% (w/w) polyoxyethylene glycerol (25) trioleate and 4% (w/w) polyoxyethylene (20) sorbitan mono-laurate together with 7.5% (w/w) ammonium nitrate
Particle size: 15 nm (mean)
Particulate size range: PSD: d99 20 nm
Silver concentration 10.0 ± 0.50% wt by assay (nominal 100mg/mL)
pH (dispersion) 7.0 – 9.0
Density 1.1 kg/dm^3
Storage condition: room temperature protected from light
Expiry date: July 2022
Purity: Specification is for an elemental silver concentration of 10.0 ± 0.50% wt (100 mg/mL).

Test item: Silver Acetate

Test item identity: Referenced as AgAc throughout the study report. AgAc: AG(I) Acetate
CAS number: 563-63-3
Intended use: Chemical feed stock
Appearance: Colourless to pale-white crystalline powder
Solubility Aqueous (room temperature): 10.2 mg/mL
Storage Conditions: Room temperature; protected from direct light
Expiry date: 22 October 2021
Purity: ≥ 99.5%

Test item: Silver Nitrate
Test item identity: Referenced as AgNO3 throughout the study report. AgNO3; Silver (I) Nitrate
CAS number: 7761-88-8
Intended use Chemical feed stock
Appearance: White crystalline powder
Solubility Aqueous (room temperature): 2560 mg/mL
Storage conditions: room temperature: protected from light
Expiry date: September 2023
Purity: 99.97 %
pH: 4.6 as 10% solution

Study summary

The study is an *in-vivo* oral route investigation using adult CD Sprague Dawley rats. The design conformed to OECD TG 417 and EU guidelines and was conducted in compliance to Good Laboratory Practice (GLP).
This study was designed as a comparative toxicokinetic assessment of the test items Silver Acetate (AgAc), Silver Nitrate (AgNO3), Micron-sized Silver (AgMP) and Nanoparticulate Silver (AgNP). This included assessment of bioavailability following single oral doses relative to single intravenous doses and systemic exposure following single and repeat oral doses. A subsidiary aim of this study was to verify that the Test Items were well tolerated in the selected toxicity species, using the chosen administration route and dosage, and to inform the dosage selection for future pivotal toxicity studies.
Groups of 4 male and 4 female CD Sprague Dawley rats were administered the test items, either as a single intravenous dose, a single oral dose or repeated daily oral doses for 4 weeks.

The study design was as follows:

Single Intravenous (bolus) and Oral Doses:

Group	Treatment	Dose (mg/kg)	Group	Treatment	Dose (mg/kg)
Phase 1.1/1.1A			Phase 2.1		
1/35	AgMP	20	7	AgMP	36
2/36	AgNP	2.5*	8	AgMP	1000
			9	AgNP	3.6*
			10	AgNP	360*
Phase 1.2			Phase 2.2		
4	AgAc	1.5	12	AgAc	5
5	$AgNO_3$	1.5	13	AgAc	175
			14	$AgNO_3$	5
			15	$AgNO_3$	125

4 week Repeat oral (gavage) Dose

Group	Treatment	Dose (mg/kg/day)	Group	Treatment	Dose (mg/kg/day)
Phase 3.1			Phase 4.1		
17	Control	0	26	Control	0
18	AgAc	5	27	AgMP	36
19	AgAc	55	28	AgMP	180
20	AgAc	175	29	AgMP	1000
Phase 3.2			Phase 4.2		
22	$AgNO_3$	5	31	AgNP	3.6*
23	$AgNO_3$	55	32	AgNP	36*
24	$AgNO_3$	125	33	AgNP	360*

* Silver equivalent value
MP Micron size particulate
NP Nanoparticulate

Blood samples were taken up to 48 hours post-dose following single intravenous and up to 96 hours post-dose following single oral doses. Blood samples were taken at pre-dose and 6 hours post-dose on Day 15 and up to 96 hours post-dose on Day 28 of daily repeat oral doses.

Selected tissues (bone marrow, brain, gastro-intestinal tract, liver, spleen, ovaries, testis and uterus) were collected at the end of the 4-week repeated dose phases.
Concentrations of silver in whole blood and tissues was measured by a validated ICP-MS method.
During the study, toxicokinetics, clinical condition, detailed physical examinations, body weight, organ weight, and macropathology investigations were undertaken.
The dose formulation analysis for the intravenous formulations of AgMP and AgNP in Phase 1.1 was unacceptable, not meeting pre-determined acceptance criteria. Modifications to the dose preparation were made and the phase repeated as Phase 1.1.A.
Several premature deaths occurred during the study. In all cases, the findings were associated with either blood sampling trauma, or dose administration trauma, and as such were considered not to be associated with treatment. Since samples from 3 other animals were available for each group on the affected occasions, there is no impact on the TK evaluation.
Sample contamination events were identified in the silver in tissue analysis segment which affected the integrity of liver and GI tract samples in Phases 3.2, Phases 4.1 and 4.2 (all other tissues in these phases were determined to be unaffected by the contamination issue). For overall study robustness reasons, the decision was made that all repeated oral

dose toxicokinetic evaluations (including silver in tissue determinations) would be re-conducted in a separate study (Labcorp study 8469368 or Charlton et al. 2021). The tissue data from these Phases 3.2, 4.1 and 4.2 are reported for completeness in the study report CC71MP or Melvin et al. 2021 but not further evaluated nor discussed.

Results:

Summary of the Silver Toxicokinetic Parameters in Rat Blood after a Single Intravenous Bolus Administration

Treatment	Dose Group	Dose Level (mg/kg)	Sex	C_{max} (ng/mL)	DN C_{max} [(ng/mL)/(mg/kg/day)]	T_{max} (h)	AUC_{0-t} (h*ng/mL)	DN AUC_{0-t} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-24} (h*ng/mL)	DN AUC_{0-24} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-inf} (h*ng/mL)	$t_{1/2}$ (h)
AgMP	35	20	M	2470	123	0.083	17100	858	8600	431	NR[a]	NR[a]
			F	1500	74.4	0.083	14300	708	7900	392	NR[a]	NR[a]
AgNP	36	2.5	M	7230	2480	0.083	14200	4860	8120	2780	NR[a]	NR[a]
			F	6070	2080	0.083	16700	5710	9980	3420	NR[a]	NR[a]
AgAc	4	1.5	M	3470	2250	0.792	70900	45900	44400	28800	NR[b]	23.6
			F	3730	2410	0.083	29800	19300	21200	13700	46700	19.0
$AgNO_3$	5	1.5	M	3220	2110	0.083	43400	28500	30400	19900	79400	17.3
			F	4130	2700	0.083	23900	15600	17100	11200	34600	19.5

All toxicokinetic parameter values are based om determination of total silver in collected whole blood

NR[a] Not reported due to an inability to characterize the elimination phase

NR[b] Not reported due to AUC extrapolated >20%

Note: AgMP and AgNP: Phase 1.1 A and AgAc and Ag NO_3: Phase 1.2

Summary of the Silver Toxicokinetic Parameters in Rat Blood after a Single Oral Gavage Administration

Treatment	Dose Group	Dose Level (mg/kg)	Sex	C_{max} (ng/mL)	DN C_{max} [(ng/mL)/(mg/kg/day)]	T_{max} (h)	AUC_{0-t} (h*ng/mL)	DN AUC_{0-t} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-24} (h*ng/mL)	DN AUC_{0-24} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-inf} (h*ng/mL)	$t_{1/2}$ (h)
AgMP	7	36	M	67.8	1.94	9.00	1690	48.5	1150	32.8	2690	28.9
			F	63.5	1.82	1.00	1730	37.5	1040	29.7	4900	28.0
	8	1000	M	71.2	0.0698	6.00	2320	2.27	1130	1.10	NR	32.2
			F	128	0.125	7.50	4320	4.23	2000	1.96	5080	30.2
AgNP	9	3.6	M	25.3	7.67	4.50	406	123	424	129	NR	22.0
			F	70.9	21.5	3.00	1130	344	841	256	3030	24.2
	10	360	M	254	0.709	9.00	11200	31.4	4500	12.6	13100	35.2
			F	296	0.828	9.00	13500	37.6	4800	13.4	13500	33.0
AgAc	12	5	M	71.6	13.3	3.50	1430	267	1050	195	3920	23.2
			F	108	20.1	2.00	2360	440	1400	260	4560	24.5
	13	175	M	367	1.95	24.0	23300	124	6320	33.6	NR	NR
			F	632	3.36	18.0	31100	130	9970	53.0	29300	25.5
$AgNO_3$	14	5	M	62.5	11.1	3.00	1550	276	994	177	NR	18.7
			F	109	19.3	3.00	4610	818	1740	309	5740	30.8
	15	125		348	2.56	24.0	19400	143	5970	43.9	19300	37.6
			F	421	3.09	12.0	18700	138	7670	56.4	19200	31.7

All toxicokinetic parameter values are based om determination of total silver in collected whole blood

NR Not reported

Note: AgMP and AgNP: Phase 2.1 and AgAc and Ag NO3: Phase 2.2

Summary of the Silver Toxicokinetic Parameters in Rat Blood after Daily Oral Gavage Administration for 4 weeks

Treatment	Dose Group	Dose Level (mg/kg/day)	Matrix	Sex	C_{max} (ng/mL)	DN C_{max} [(ng/mL)/(mg/kg/day)]	T_{max} (h)	AUC_{0-t} (h*ng/mL)	DN AUC_{0-t} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-24} (h*ng/mL)	DN AUC_{0-24} [(h*ng/mL)/(mg/kg/day)]	$t_{1/2}$ (h)
AgAc	18	5	Blood	M	153	43.4	7.50	6220	1770	2610	741	32.1
				F	216	61.7	3.00	10200	2920	3950	1130	41.3
AgAc	19	55	Blood	M	404	11.3	6.00	23800	664	8160	228	NR[a]
				F	477	13.3	6.00	30200	846	10000	280	46.8
AgAc	20	175	Blood	M	766	6.09	3.00	43000	342	15200	121	36.3
				F	930	7.29	3.00	51000	400	19400	152	40.4

All toxicokinetic parameter values are based om determination of total silver in collected whole blood

NR[a] Not reported due to an inability to characterize the elimination phase

Note: AgAc: Phase 3.1

Mean concentrations (ng/mL) of silver in tissues following repeated oral doses of AgAc

Group /Sex		Bone marrow	Brain	GI tract	Spleen	Testis	Liver
17M	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
18M	Mean	62.0	142	2770	283	168	55.4
	SD	13.5	36.0	1720	71.2	75.9	15.3
19M	Mean	3500	637	19200	38700	1510	9470
	SD	1540	57.2	7680	11000	215	4120
20M	Mean	21400	1460	83200	96400	1530	22700
	SD	15400	33.1	5270	55200	237	7630

Group /Sex		Bone Marrow	Brain	GI Tract	Spleen	Ovaries	Uterus	Liver
17F	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
18F	Mean	126	169	3800	990	2200	188	305
	SD	110	21.2	1490	455	1080	121	232
19F	Mean	4500	805	50700	60800	24300	8000	16500
	SD	1420	35.9	11200	10400	2610	3300	3610
20F	Mean	46800	1460	104000	142000	39700	11100	21900
	SD	19800	114	12600	93500	19800	2320	13100

BLQ <5.00 ng/mL

As mentioned in Annex 3 of this document: "*Given that absolute bioavailability (F-values) had only been reported in the literature for a few silver compounds and nanosilver substances, a conventional single dose bioavailability assessment was attempted for all the test items – refer to Report Number CC71MP. As mentioned in that report (Section 8), anomalous results were obtained in the case of the silver powder (AgMP) relating to the blood concentration profiles which were evident following intravenous bolus administration. The derived F-value was clearly incompatible with the observed AUC data via the oral route after a single dose. Furthermore, separately performed mass-balance calculations indicated that very rapid clearance of AgMP had occurred from the blood compartment.*

An explanation for the artefactually elevated F-value results for AgMP has not yet been fully elucidated. However, it is established in the scientific literature that certain micron-size particulates can be very rapidly sequestered by the reticuloendothelial system when administered via the i.v. route. We cannot exclude the possibility that the F-value derived for the suspension particulate AgNP test item may also have been subject to some artefactual distortion.

In alignment with the direction taken by the CRO investigator, comparative TK assessment of the various Ag test items should instead be based on their respective p.o. dose-normalised AUC group mean values (as an indicator of relative systemic exposure) – refer to the report summary sections. Further insights can also be obtained by comparison of AUC profiles obtained after repeated oral dosing."

Conclusion:

The data suggest that following administration of AgAc over a 28-day period, the test item was tolerated over the range 5 – 175 mg/kg/day, however, this is based on bodyweight, clinical and macropathogical observation; no other toxicological endpoints were evaluated.

Generally systemic exposure to silver in females tended to be slightly higher than that in males, and this trend was reflected in the tissue concentrations after repeated doses of AgAc.

Systemic exposure, as assessed by mean C_{max} and AUC0-24 values, increased with dose level after single oral doses of AgMP at dose levels of 36 and 1000 mg/kg; AgNP at dose levels of 3.6 and 360 mg/kg; AgAc at dose levels of 5 and 175 mg/kg; and AgNO3 at dose levels of 5 and 125 mg/kg. These increases in systemic exposure were less than dose proportional and this was particularly notable for AgMP. The increase in systemic exposure to silver after repeated oral doses of AgAc over the dose range 5 - 125 mg/kg/day was less than proportional.
After repeated oral doses of AgAc, there was evidence of accumulation in the systemic exposure and comparison of pre-dose and 6-hour post-dose concentrations on Days 15 and 28 indicate steady state had been reached by Day 15.

After repeated oral doses of AgAc measurable concentrations of silver were found in all tissues examined. Tissue concentrations increased with dose, but these increases were not proportional. In the bone marrow, liver, spleen and uterus these increases were greater than proportional, however, in the brain, GIT, testis and ovaries these increases were less than proportional.

The findings in this study have demonstrated that after single oral doses of the test items, the systemic exposure to silver can be ranked in the following order AgAc/$AgNO_3$ >> AgNP >>> AgMP.

5 DETAILED STUDY SUMMARY AND RESULTS OF CHARLTON ET AL., 2021 STUDY

Silver Nitrate, Micron-sized Silver and Nanoparticulate Silver: A Comparative Toxicokinetic Study in Rats by Repeat Administration. Labcorp Study Number: 8469368

Information substance identity

Test item: Micron-sized Silver
Test item identify: Reference das AgMP throughout the study report
Alternative names: AgMP; Bulk Silver; Bulk Ag; Micron-sized silver particulate; Silver powder and silver flake
CAS number 7440-22-4
Intended use: Chemical feed stock
Appearance Silver/grey powder
Composition: Uncoated silver particulate (crystalline Ag powder of highly uniform spheroidal shape)
Particulate size ~ 0.4 µm
Particulate size range: PSD: d90; 0.57 µm / d50; 0.36 µm / d10; 0.25 µm
Density (tapped): 3.8 kg/dm^3
Storage condition: Room temperature: protected from light
Expiry date: 29 March 2022
Purity: > 99%

Test item: Nanoparticulate Silver
Test item identity: Referenced as AgNP throughout the study report
Alternative names: AgNP, nanoAg, nanosilver
CAS number: 7440-22-4
Intended use: Chemical feed stock
Appearance: Dark orange liquid
Molecular mass: 107.87 g/mol
Composition: Surfactant stabilized aqueous dispersion of nanoparticles containing 4% (w/w) polyoxyethylene glycerol (25) trioleate and 4% (w/w) polyoxyethylene (20) sorbitan mono-laurate together with 7.5% (w/w) ammonium nitrate
Particle size: 15 nm (mean)
Particulate size range: PSD: d99 20 nm
Silver concentration 10.0 ± 0.50% wt by assay (nominal 100 mg/mL)
pH (dispersion) 7.0 – 9.0
Density 1.1 kg/dm^3
Storage condition: room temperature protected from light
Expiry date: February 2024
Purity: Specification is for an elemental silver concentration of 10.0 ± 0.50% wt (100 mg/mL).

Test item: Silver Nitrate
Test item identity: Referenced as AgNO3 throughout the study report. AgNO3; Silver (I) Nitrate
CAS number: 7761-88-8
Intended use Chemical feed stock
Appearance: White crystalline powder
Molecular mass: 169.87 g/mol (or which 107.87 g/mol Ag)
Solubility Aqueous (room temperature): 2560 mg/mL
Storage conditions: room temperature: protected from light
Expiry date: September 2023
Purity: 99.97 %
pH: 4.6 as 10% solution

Study summary and results

Information concerning the toxicokinetics of the test items Silver Nitrate (AgNO3), Micron-Sized Silver Powder (AgMP) and Silver Nanoparticles (AgNP), in the Sprague Dawley rat was required to support toxicology studies in animals. A subsidiary aim of this study was to verify that the test item was well tolerated in the selected toxicity species, using the chosen administration route and dosage, and to inform the dosage selection for future pivotal toxicity studies.

The study design was as follows:

Phase	Group	Treatment	Route	Dose (mg/kg/day)	Number of animals/sex
1	1	Control	Oral gavage	0	4M + 4F
	2	AgMP		36	4M + 4F
	3	AgMP		180	4M + 4F
	4	AgMP		1000	4M + 4F
2	6	Control	Oral gavage	0	4M + 4F
	7	AgNP		3.6*	4M + 4F
	8	AgNP		36*	4M + 4F
	9	AgNP		360*	4M + 4F
3	11	Control	Oral gavage	0	4M + 4F
	12	$AgNO_3$		5	4M + 4F
	13	$AgNO_3$		55	4M + 4F
	14	$AgNO_3$		125	4M + 4F

MP Micron-sized silver powder
NP Silver Nanoparticles
* Silver equivalent value

Blood samples were taken at pre-dose and 6 hours post-dose on Day 15 and up to 96 hours post-dose on Day 28 of daily repeat oral doses.
Selected tissues (bone marrow, brain, gastro-intestinal tract, liver, spleen, ovaries, testis and uterus) were collected at the end of the 4-week repeated dose phases.
At termination, analysis of serum for total copper concentration was undertaken in the case of the test items AgMP and AgNO3.
Concentrations of silver in whole blood and tissues was measured by a validated ICP-MS method. During the study, toxicokinetics, clinical condition, detailed physical

examinations, body weight, organ weight, and macropathology investigations were undertaken.

Results

Mean concentrations of silver in tissues following repeated oral doses of AgMP for 28 days.

Group /Sex		Bone marrow	Brain	GI tract	Spleen	Testis	Liver
1M	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC
2M	Mean	BLQ	76.0	261	77.5	76.8	BLQ
	SD	NC	17.7	134	31.4	36.0	NC
3M	Mean	NC	117	1004	164	213	64.3
	SD	NC	47.0	1271	141	180	30.2
4M	Mean	NC	159	2620	516	296	48.7
	SD	NC	26.0	721	195	80.6	9.33

Group /Sex		Bone Marrow	Brain	GI Tract	Spleen	Ovaries	Uterus	Liver
1F	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC	NC
2F	Mean	BLQ	75.6	310	89.8	81.9	BLQ	77.9
	SD	NC	16.1	162	58.7	31.8	NC	34.3
3F	Mean	BLQ	166	3755	1006	1328	153	405
	SD	NC	34.9	1853	623	917	58.8	619
4F	Mean	243	187	5124	1448	2470	184	274
	SD	89.3	27.6	1645	830	1273	127	186

BLQ <5.00 ng/mL
N Not calculated

Mean concentrations of silver in tissues following repeated oral doses of AgNP for 28 days

Group /Sex		Bone marrow	Brain	GI tract	Spleen	Testis	Liver
6M	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC
7M	Mean	BLQ	77.5	156	77.0	66.1	BLQ
	SD	NC	13.9	42.8	38.6	26.6	NC
8M	Mean	181	226	3888	938	452	154
	SD	107	46.5	1799	920	173	114
9M	Mean	NC	575	11466	14928	1310	1949
	SD	NC	136.9	8252	5257	152	900

Group /Sex		Bone Marrow	Brain	GI Tract	Spleen	Ovaries	Uterus	Liver
6F	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC	NC
7F	Mean	BLQ	64.8	415	53.2	116	BLQ	77.2
	SD	NC	12.5	196	21.8	93.7	NC	50.4
8F	Mean	NC	221	5784	2233	5957	225	382
	SD	NC	13.4	726	1069	2255	133	233
9F	Mean	NC	604	9630	28081	19170	3865	4325
	SD	NC	36.8	3721	7067	6142	1915	1544

BLQ <5.00 ng/mL
NC Not calculated

Mean concentrations of silver in tissues following repeated oral doses of $AgNO_3$ for 28 days

Group /Sex		Bone marrow	Brain	GI tract	Spleen	Testis	Liver
11M	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC
12M	Mean	73.1	142	1310	404	231	81.7
	SD	16.2	35.4	214	191	103	33.1
13M	Mean	10739	632	5319	39789	1202	4668
	SD	2104	129	2412	5704	246	2919
14M	Mean	46553	1091	10592	54070	1464	28859
	SD	26985	57.0	10669	21236	260	36648

Group /Sex		Bone Marrow	Brain	GI Tract	Spleen	Ovaries	Uterus	Liver
11F	Mean	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
	SD	NC	NC	NC	NC	NC	NC	NC
12F	Mean	87.0	177	2438	722	1484	145	130
	SD	36.8	41.7	425	335	561	81.7	39.8
13F	Mean	NC	700	3624	41558	31357	8104	5295
	SD	NC	96.2	1449	5668	8793	3075	3561
14F	Mean	25200	1019	14798	62335	44025	22466	16024
	SD	4630	209	7728	22794	16158	4327	6464

BLQ <5.00 ng/mL
NC Not calculated

Summary of the Silver Toxicokinetic Parameters in Rat Blood after Daily Oral Gavage Administration for 4 weeks (Day 28)

Treatment	Dose Group	Dose Level (mg/kg/day)	Sex	C_{max} (ng/mL)	DN C_{max} [(ng/mL)/(mg/kg/day)]	T_{max} (h)	AUC_{0-t} (h*ng/mL)	DN AUC_{0-t} [(h*ng/mL)/(mg/kg/day)]	AUC_{0-24} (h*ng/mL)	DN AUC_{0-24} [(h*ng/mL)/(mg/kg/day)]	$t_{1/2}$ (h)
AgMP	2	36	Male	73.3	2.21	6.00	2740	82.4	1270	38.2	29.3
			Female	125	3.76	4.50	4850	145	2110	63.3	36.6
AgMP	3	180	Male	123	0.743	4.50	5980	36.1	2360	14.2	33.4
			Female	192	1.15	3.00	8550	50.9	3620	21.6	41.4
AgMP	4	1000	Male	139	0.172	4.50	7130	8.85	2730	3.39	31.5
			Female	229	0.280	6.00	10700	13.1	4390	5.39	43.4
AgNP	7	3.6	Male	62.0	17.2	6.00	2470	686	1210	337	31.7
			Female	90.8	25.1	3.00	3900	1080	1560	431	36.4
AgNP	8	36	Male	150	3.95	6.00	7070	186	2910	76.6	36.9
			Female	278	7.22	6.00	10600	276	4530	118	38.9
AgNP	9	360	Male	299	0.786	9.00	13400	35.3	5460	14.3	42.4
			Female	485	1.27	10.5	25700	67.3	9940	26.0	40.7
$AgNO_3$	12	5	Male	132	38.6	4.50	5470	1600	2290	670	42.1
			Female	177	51.7	6.00	7690	2240	3220	940	37.5
$AgNO_3$	13	55	Male	413	10.6	12.0	22600	581	8260	212	NR
			Female	483	12.5	10.5	23300	601	9450	244	45.0
$AgNO_3$	14	125	Male	738	8.21	12.0	42800	476	14900	166	42.0
			Female	997	11.1	18.0	54100	601	20400	227	39.8

NR Not reported due to an inability to characterize the elimination phase

Note: AgMP: Phase 1, AgNP: Phase 2 and $AgNO_3$: Phase 3

All toxicokinetic parameter values are based on determinations of total silver in collected whole blood.

Conclusion:

The data suggest that following administration of AgMP (range 36 to 1000 mg/kg/day), AgNP (range 3.6 to 360 mg/kg/day) and $AgNO_3$ (range 5 to 125 mg/kg/day) over a 28-day period, the test items were well tolerated (based on clinical and macro-pathological observations, together with body weight).

Systemic exposure, as assessed by silver mean C_{max} and AUC0-t values, increased with the increase in AgMP dose level from 36 to 1000 mg/kg/day, the increase in AgNP dose level from 3.6 to 36 mg/kg/day and the increase in AgNO3 dose level from 5 to 125 mg/kg/day.
These increases in mean C_{max} and AUC0-t were less than dose proportional and this was particularly notable for AgMP.

Concentrations of silver in tissues generally increased with dose, however, there was no consistent relationship, and the dose response was not a simple monotonic characteristic.

Systemic exposure and concentrations of silver in tissues were slightly higher in females than in males.

After repeated oral doses, measurable concentrations of silver were found in all tissues, except for the liver in males after low doses of AgMP and AgNP and in the uterus in females at the low dose of AgMP. The highest concentrations following doses of AgMP and AgNP were found in the gastro-intestinal tract, accounting for 40-70 % of the total amount of silver. Highest concentrations following oral doses of $AgNO_3$ were generally found in the spleen and bone marrow. Lowest concentrations were generally found in the brain and testis.

The findings in this study have demonstrated that after repeated oral doses of the test items the systemic exposure to silver can be ranked in the following order AgNO3 > AgNP > AgMP.

There was no treatment-related effect on the concentrations of copper in serum following repeated oral doses of AgMP, however, after repeated oral doses of $AgNO_3$, the serum concentrations of copper decreased with increasing dose.

6 ANNEXES

<u>Annex 1:</u> Deviation from study plan of study Melvin P et al. (2021)

<u>The following deviations from study plan occurred:</u>

The intravenous trial formulation of AgNP was prepared at 0.05 mg/mL instead of 0.5 mg/mL. As storage temperature was revised from ambient to refrigerated due to refinement of preparation these samples became redundant and were not analysed.

Sampling during formulation trials: The trial oral formulations of AgNP, were sampled on Day 16 instead of Day 15.

Blood sampling, Phase 1.2, Group 5F, animals 218, 12-hour time point; the sample was taken at 00:13 hours and was found the following day at 15:15 hours on the roller. This sample should have been snap frozen and transferred to -20°C storage.

Higher than expected achieved concentration results were obtained during formulation analysis of the AgMP intravenous formulation in Phase 1.1. The verification weights suggest 1.1 mL was consistently taken for these formulation samples rather than 1 mL. Note this phase was replaced with Phase 1.1A.

During dose formulation sampling for AgAc vehicle (control group) on Day 1, the technician did not add the required volume of acid to bring the sample to the required total volume and reference pH range.

Phase 4.1, Groups 26-29 the dose formulation was stored at the incorrect temperature (stored ambient instead 4°C). Since this phase will be repeated, the validity of this study is not impacted.

Phase 3.2, blood sampling of animal 275 at the 3-hour timepoint on Day 28 (Phase 3.2) was sampled 3 minutes late at 183 minutes post dose.

The following deviations from SOP occurred and are not expected to have affected the integrity or outcome of the study:

Phase 2.2; Animals 253 and 254 at the 3-hour timepoint were bled outside the SOP limits, 2 minute deviation.

Phase 3.1, repeat oral dose of AgAc, on Day 11 dose accounting was outside of SOP limits. Groups 19 and 22 were – 1.1% under the limit and Group 20 was 13.1% over the limit.

On 29 November 2020, the humidity rose to 72%, outside limit of 70%, for a transient period.

On the 30 November 2020, the temperature of the cold store used for storing dose formulations (Phases 3.1 and 4.1) in the animal unit rose outside the SOP limits of 2 - 8°C, riding to 16°C for less than 1 hour.

Humidity dropped to 37% from 18:00 hours on 29 December 202 until 10:00 hours 30 December.

Annex 2

Deviation from study plan of study Charlton et al. (2021)

The following deviations from study plan occurred:
On Day 18 the 3-4-hour observations were not performed for Phase 3 animals. At the 6-hour and end of day observation the animals were recorded within normal limits.

The missed observation at 3-4 hours after dosing on Day 18 for Phase 3 animals had no impact on the study since observations at 1-2 hours and 6 hours after dosing confirmed animals were within normal limits. There was no evidence to suggest that the animals were not within normal limits at the missed time point.

Formulation sample results received for AgMP for Group 2-4 from Week 4 were lower than expected (-60% versus expected nominal concentration). It was observed that the samples received by the Principal Investigator for analysis for these groups were cloudy in appearance and not clear solutions as expected. It was deemed that the samples taken were incorrectly stabilized with dilute rather than concentrated nitric acid. Since dosing was already underway using the Week 4 preparation, re-sampling was only possible to be performed on five remaining aliquots intended for use on 05-09 August 2021. Aliquots were combined and resuspended prior to additional sampling. A second set of samples were taken (properly stabilized) and sent for analysis as the Week 4 formulations.

The associated analytical results received were low for all three groups (refer to Section 5.1). Group 2 and 3 results were -8.15% and -7.83% respectively versus nominal concentration; these being within acceptance limits. The Group 4 result was determined as -19.1% below the nominal concentration. This value lies outside the normally applied acceptance criterion of 100±15% recovery with ≤ 10% RSD for suspension formulations. It is considered that this analytical outcome was most likely due to the resampling procedure and associated limitations in only sampling a portion of the total formulation which was originally prepared. Given these circumstances and the relatively small deviation from the acceptance criterion, it is not considered that the sampling deviation event had a detrimental impact on overall study integrity.

A blood sample was not achieved for Animal 11M, Group 3 on Day 28 at the 72 hour timepoint and for Animal 32M, Group 9 on Day 28 at the 96 hour time-point. There is no impact on toxicokinetic evaluation due to these deviations since sufficient data points from at least 2 other animals were available for each of the affected groups.

During centrifugation, the GIT tissue sample was lost for Animal 111F, Group 3, during the digestion process. There is no impact on tissue evaluation since sufficient data from 3 other animals were available for the affected group.

Terminal blood collections for copper determination in serum of animals from Phases 1 and 3 were taken via the sublingual vein as an alternative suitable site, the use of isoflurane was not specified in the Study Plan as the anesthetic that was to be used. This

had no impact on the samples since the principal route of collection would have used isoflurane.

Unscheduled animal death

Animal number 34 was found dead after being dosed on Day 11. The necropsy report confirmed the animal had a perforated oesophagus. This finding is considered consistent with dose administration trauma and not associated with treatment with the test substance.

The following deviations from SOP occurred and are not expected to have affected the integrity or outcome of the study:

On Day 8 the Group 13 expected weight was 49.6 g and the actual weight used was 49.0 g, therefore the total % was -1.2% = 0.2% under the 1% limit.

On Day 9 the Group 11 expected weight was 50.0 g and the actual weight used was 48.0 g, therefore the total % was -4.0% = 3.0% under the 1% limit.

On Day 11 the Group 12 expected weight was 48.4 g and the actual weight used was 47.6 g, therefore the total % was -1.6% = 0.6% under the 1% limit.

On Day 13 the Group 7 expected weight was 48.2 g and the actual weight used was 47.7 g, therefore the total % was -1.0% = 0.0% under the 1% limit.

On Day 14 the Group 12 expected weight was 48.4 g and the actual weight used was 47.6 g, therefore the total % was -1.7% = 0.7% under the 1% limit.

On Day 15 the Group 12 expected weight was 52.2 g and the actual weight used was 51.5 g, therefore the total % was -1.3% = 0.3% under the 1% limit.

On Day 15 the Group 13 expected weight was 51.6 g and the actual weight used was 50.8 g, therefore the total % was -1.6% = 0.6% under the 1% limit.

On Day 15 the Group 14 expected weight was 50.2 g and the actual amount used was 57.5 g therefore the total % used was 14.5%= 4.5% over the 10% limit.

On Day 18 the Group 6 expected weight was 51.8 g and the actual weight used was 50.6 g, therefore the total % was -2.3% = 1.3% under the 1% limit.

On Day 18 the Group 7 expected weight was 51.8 g and the actual weight used was 51.0 g, therefore the total % was -1.5% = 0.5% under the 1% limit.

On Day 18 the Group 8 expected weight was 51.4 g and the actual weight used was 50.4 g, therefore the total % was -1.9% = 0.9% under the 1% limit.

On Day 18 the Group 12 expected weight was 52.2 g and the actual weight used was 51.6 g, therefore the total % was -1.1% = 0.1% under the 1% limit.

On Day 19 the Group 2 expected weight was 53.8 g and the actual weight used was 53.1 g, therefore the total % was -1.3% = 0.3% under the 1% limit.

On Day 19 the Group 11 expected weight was 53.4 g and the actual weight used was 52.8 g, therefore the total % was -1.1% = 0.1% under the 1% limit.

On Day 20 the Group 11 expected weight was 53.4 g and the actual weight used was 52.8 g, therefore the total % was -1.1% = 0.1% under the 1% limit.

On Day 20 the Group 12 expected weight was 52.2 g and the actual weight used was 51.6 g, therefore the total % was -1.1% = 0.1% under the 1% limit.

On Day 21 the Group 2 expected weight was 53.8 g and the actual weight used was 52.8 g, therefore total % was -1.9% = 0.9% under the 1% limit.

On Day 21 the Group 3 expected weight was 52.8 g and the actual weight used was 52.0 g, therefore the total % was -1.5% = 0.5% under the 1% limit.

On Day 21 the Group 8 expected weight was 51.4 g and the actual weight used was 50.8 g, therefore the total % was -1.2% = 0.2% under the 1% limit.

On Day 22 the Group 12 expected weight was 52.2 g and the actual weight used was 51.6 g, therefore the total % was -1.1% = 0.1% under the 1% limit.

On Day 23 the Group 1 expected weight was 55.6 g and the actual weight used was 54.8 g, therefore the total % was -0.8% = 1.4% under the 1% limit.

On Day 24 the Group 3 expected weight was 55.0 g and the actual weight used was 54.3 g, therefore total % was -1.3% = 0.3% under the 1% limit.

On Day 24 the Group 12 expected weight was 54.2 g and the actual weight used was 53.5 g, therefore the total % was -1.3% = 0.3% under the 1% limit.

On Day 25 the Group 6 expected weight was 52.2 g and the actual weight used was 54.1 g, therefore the total % was -1.9% = 0.9% under the 1% limit.

On Day 26 the Group 1 expected weight was 55.6 g and the actual weight used was 54.9 g, therefore the total % was -0.7% = 1.3% under the 1% limit.

Annex 3

INFORMATION TRANSMITTAL – 15/12/2021
Comparative toxicokinetics (TK investigations of different forms of silver (Ag): Final report transfers

Executive summary

The EPMF comparative TK study is a high quality GLP investigation and the first which permits a direct comparison of soluble silver compounds, nanosilver and silver powder (tested as a sub-micron sized powder-form) – in terms of relative oral uptake and tissue distribution of silver.

- The study consists of two study reports and the findings obtained for the different forms of silver across the two study segments can be compared.
- Comparison of AUC profiles obtained after repeated oral dosing confirm that **oral intake of silver powder results in markedly lower systemic exposure than seen for soluble silver compounds and nanosilver**.
- When looking at differentials in silver in tissue concentrations following repeated dosing, for the majority of tissues, **exposure to soluble silver compounds leads to tissue Ag levels which are 2 to 3 orders of magnitude greater than those evident for the silver powder**. The tissues for which the difference is less than 2 to 3 orders of magnitude, are minor sites of distribution.
- The study shows that the **testis** and **brain** are minor sites of distribution. In contrast, the **ovary** has been shown to be an important site of distribution. However, ancillary investigations strongly suggest silver is present in the ovary as chemically stable silver sulphide/selenide complexes with very low local bioavailability.
- The study findings strongly suggest that the **direct read-across of mammalian toxicity datasets for soluble silver compounds (like silver acetate) and nanosilver to silver powder is not supported** (from a scientifically valid toxicokinetic perspective).

1. Reporting of TK study findings

As previously notified by EPMF to ECHA, a technical failure during the conduct of the Ag in tissue analysis necessitated a repeat of one part of the programme relating to the micron-sized silver powder (AgMP), nanosilver (AgNP) and silver nitrate test items[1]. As a result, the findings for single dose investigations on all test items together with the repeat dose phase outcomes for silver acetate are covered in Report Number CC71MP; with all other repeat dose phase findings being addressed in Report Number 8469368. An official statement released by the originating contract laboratory[2] confirms that both parts of the overall programme were performed to an exactly equivalent design and under matched experimental conditions. Hence, we believe it is scientifically justifiable to compare findings obtained for the different forms of silver across the two study segments.

2. Investigation of absolute bioavailability (F-value)

Given that absolute bioavailability (F-values) had only been reported in the literature for a few silver compounds and nanosilver substances, a conventional single dose bioavailability assessment was attempted for all the test items – refer to Report Number CC71MP. As mentioned in that report (Section 8), anomalous results were obtained in the case of the silver powder (AgMP) relating to the blood concentration profiles which were evident following intravenous bolus administration. The

[1] An analytical cross-contamination event called into question the integrity of data for two of the investigated tissues (GI tract and liver) relating to the AgMP, AgNP and $AgNO_3$ test items. All other tissues obtained were not affected by this technical issue. However, to assure the absolute integrity of the overall TK program, a decision was made to repeat all repeat dose phase work (i.e., Ag in tissue and Ag in blood) for these test items. This necessitated separate reporting.

[2] Annex 3_Dec2021_ECHA Letter_EPMF_TK Study Final, letter from Labcorp dated 14 Dec 2021, signed by Mark Terry.

derived F-value was clearly incompatible with the observed AUC data via the oral route after a single dose. Furthermore, separately performed mass-balance calculations indicated that very rapid clearance of AgMP had occurred from the blood compartment.
An explanation for the artefactually elevated F-value results for AgMP has not yet been fully elucidated. However, it is established in the scientific literature that certain micron-size particulates can be very rapidly sequestered by the reticuloendothelial system when administered via the i.v. route. We cannot exclude the possibility that the F-value derived for the suspension particulate AgNP test item may also have been subject to some artefactual distortion.
In alignment with the direction taken by the CRO investigator, comparative TK assessment of the various Ag test items should instead be based on their respective p.o. dose-normalised AUC group mean values (as an indicator of relative systemic exposure) – refer to the report summary sections. Further insights can also be obtained by comparison of AUC profiles obtained after repeated oral dosing.

3. Updated toxicokinetics comparisons for the various Ag forms

(a) Achieved systemic exposure (Ag in blood) followed repeated dosing

Based on the reported datasets we have updated the previously forwarded plots of $AUC_{0\text{-}t}$ for all the respective Ag test items (Figure 1), clearly showing the TK difference between AgMP and the other test items. We would respectfully remind you that the AgMP reference selected for investigation had a median particle size of ca. 0.3 µm (identified as the smallest representative silver powder form put on the market by EPMF members). We consider this particle size represents a worst-case type of silver powder in terms of expected bioavailability.

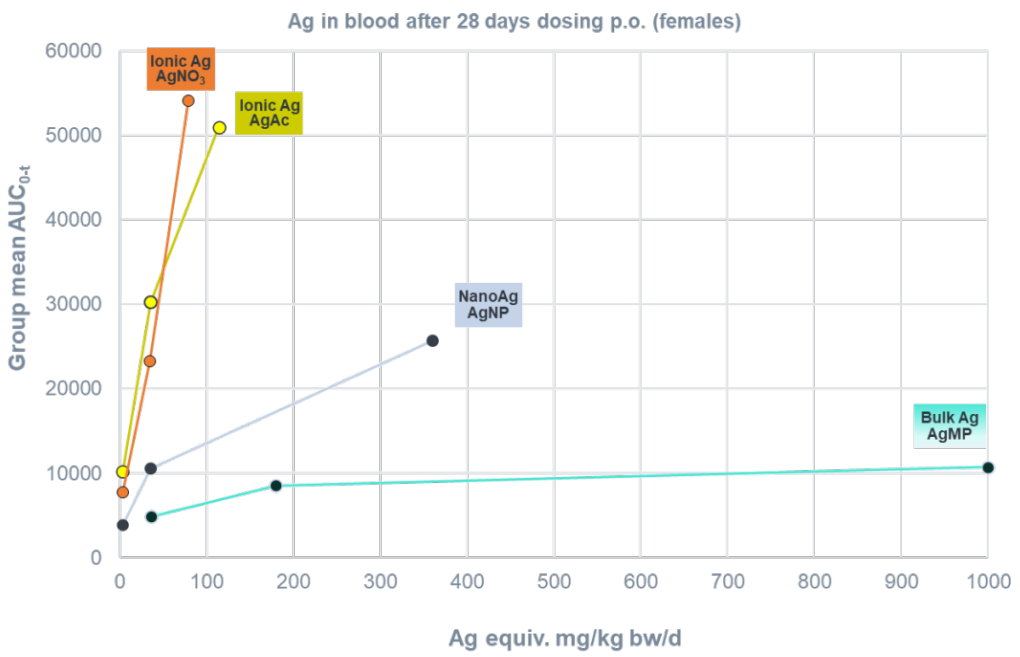

Figure 1. Achieved systemic exposure for soluble silver compounds (silver nitrate (AgNO3) and silver acetate (AgAc)), nanosilver (AgNP) and silver powder (AgMP).
Results depicted are for female animals (very similar findings were obtained for males).

(b) Differentials in Ag in tissue concentrations following repeated dosing

When predicting the potential for toxicity for forms of Ag with variable bioavailability, it is reasonable to also consider differentials in key tissue/target organ exposure levels. As an example, and adopting the most conservative approach, such a comparison may be based on the attained tissue Ag concentrations arising from the highest administered/tolerated dose of Ag in respect of a soluble silver compound versus that for a Ag powder form. In the case of the latter, this represents the tissue exposure attained at a Limit Dose of 1000 mg Ag/kg bw/d

administered via the oral route for a period of 4 weeks[3]. We have performed such a data analysis in Table 1 (using AgAc as principle ionic reference). It may be seen that for the majority of tissues, exposure to the soluble silver compound leads to tissue Ag levels which are 2 to 3 orders of magnitude greater than those evident for the silver powder. In confirmation of the markedly different TK characteristics of soluble silver compounds, similar differentials are apparent when comparing Ag in tissue levels attained at the maximally administered doses of silver nitrate versus AgMP.

	Achieved tissue concentrations expressed as DN basis (ng Ag per gram tissue / Ag dose)												
	GI tract		Liver		Spleen		Brain[1]		Bone marrow		Ovary	Uterus	Testis[1]
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀			
AgAc Group Mean [Ag] in tissue (ng/g) / Dose (mg Ag/kg bw/d)	730	912	199	192	846	1246	13	13	188	410	348	97	13
AgMP Group Mean [Ag] in tissue (ng/g) / Dose (mg Ag/kg bw/d)	2.62	5.12	0.05	0.27	0.52	1.45	0.16	0.19	NC	0.24	2.47	0.18	0.27
AgAc : AgMP Fold-difference comparing DN values	279	178	3980	711	1627	859	80	67	NC	1708	141	541	45

AgAc highest dose level: 175 mg AgAc/kg bw/d [equivalent to 114 mg Ag/kg bw/d].
AgMP highest dose level: 1000 mg Ag/kg bw/d.
NC = Not calculable due to detection limit DN = Dose normalised
Nota 1: Tissue which exhibits clear evidence of Ag uptake kinetics saturation.

Table 1. Differentials in achieved tissue concentrations of Ag. Comparison of a soluble Ag compound (silver acetate; AgAc) with silver powder (AgMP)

(c) Remarks on tissue distribution profile (male and female reproductive tissues and CNS)

The Summary sections of the reports include data tables of tissue Ag concentrations determined after treatment with the respective test items. When data from all dose levels are considered in terms of absolute tissue Ag levels, it is evident that the testis and brain are minor sites of distribution (especially in the case of ionic Ag references). There is also evidence of plateauing of Ag concentrations in these tissues as the dose level increases. This would be expected if blood-testis and blood-brain barriers operate to limit uptake of Ag+. Similar saturation kinetics, as well as the identification of these tissues as only minor sites of distribution, have been shown in several previous TK studies in rodent models (using both oral and parenteral routes of administration)4 (Lansdown 2007, Loeschner et al. 2011, Lankveld et al. 2010, Kim et al. 2008, Pang et al. 2016, Juling et al. 2016).
Given the significant vascular compartment associated with the brain, it should not be assumed that all the detected Ag in that organ resides within the parenchyma. Indeed, previously published studies (typically based on autometallography as a technique, e.g. Rungby and Danscher 1983a & 1983b), as well as recent ancillary work by EPMF, demonstrates that much of the identifiable Ag depots are associated with the CNS endothelial sub-compartment (given that Ag binds to endothelial cell membranes) or are deposited in basal lamina of the choroid plexus.

In contrast, it is acknowledged that the ovary has been shown to be an important site of distribution5; -- in terms of relative rank order of tissue distribution for all the test items, and due to the notable absolute Ag concentrations observed for both soluble Ag substances and AgNP. We believe the basis for this finding is that the ovary is known to contain relatively high levels of sulphur and selenium (undergoing more rapid turnover than in some other tissues) (Boja et al. 2005, Ceko et al. 2016, Thomson et al. 1975). Such an intra-organ environment favours sequestration of systemically available Ag+, via its conversion into Ag sulphur/selenide

[3] The submitted TK reports confirm that steady state kinetics is evident following oral dosing for approximately 2 weeks, i.e., in keeping with other literature reports relating to ionic Ag substances and also AgNP.

[4] Due to the uptake saturation effect, certain other TK investigations based only on administration of low doses of Ag have erroneously concluded that the CNS and/or testis are significant sites of distribution.

[5] Based on the published literature, the EPMF program is the first systematic investigation to examine Ag toxicokinetics for the ovary.

complexes. However, as with all other situations of argyria, it is important to bear in mind that the Ag sulphur/selenide complexes possess ultralow solubility (Ksp values of ~10^{-50} to 10^{-65}) and are chemically stable with very low local bioavailability when formed in tissues. They have been identified as key chemical species in tissue argyria, a phenomenon commonly recognised as a key mammalian detoxification mechanism for the Ag ion (Lansdown 2010, Aaseth et al. 1981, Berry and Galle 1982).

EPMF have performed ancillary investigations on ovarian tissue obtained from rats treated with silver acetate in order to obtain information on the speciation of the Ag depots. The work[6], which was based on Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy (SEM-EDX) characterisation directed by autometallography (AMG) localisation, has confirmed that the ovarian Ag depots are co-located with sulphur and selenium in the form of 250-500 nm sized structures in the tissue (example micrographs provided in Figure 2). This type of co-location pattern is strongly indicative of the existence of Ag sulphide/selenide complexes. Further support for this conclusion arises from tissue selenium determinations. These show that treatment with ionic silver elevates total ovarian Se levels markedly above control values[7] -- presumably due to the trapping of Se in the immobile complexes. In relation to the detected ovarian Ag levels, it is appropriate not only to consider quantitative distributional toxicokinetics of Ag but also the speciation context.

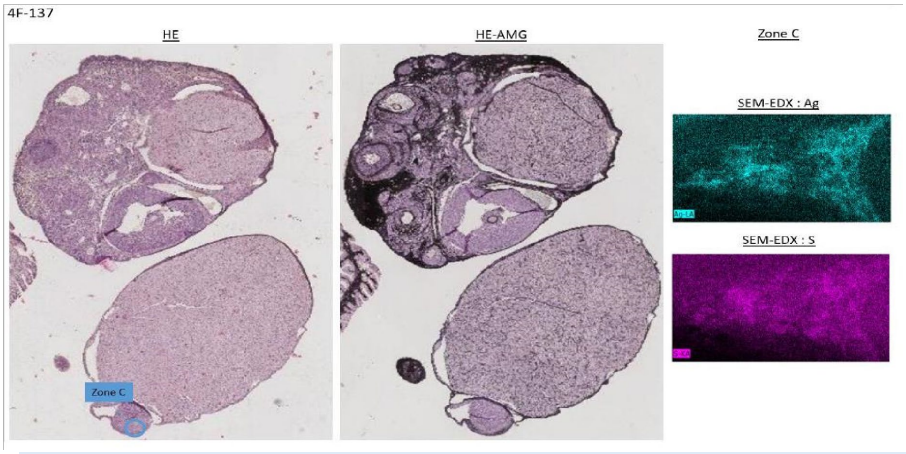

Figure 2. Representative micrographs of an ovarian tissue section from an animal which received repeated p.o. doses of AgAc[6].

From L to R: Optical microscopy (H&E staining); Ag-AMG image; SEM-EDX images with element cartography.

[6] Lison, D. and Ryelandt, L. (2021) Scanning electron microscopy (SEM)-energy dispersive X-ray (EDX) studies of ovarian tissue obtained from rats treated with a form of ionic silver (silver acetate). LTAP, University of Louvain.

[7] Silver Acetate: Preliminary reproductive performance study in the Sprague Dawley Rat by dietary administration (8436495): Bioanalysis Phase Report – Copper and Selenium in tissue. Arcinova Draft report, 2021.

References

Aaseth J, Olsen A, Halse J, Hovig, T (1981) Argyria—tissue deposition of silver as selenide. Scand J Clin Lab Invest. 41, 247–251.

Berry JP, Galle P (1982) Selenium and kidney deposits in experimental argyria: Electron microscopy and microanalysis. Pathologie Biologie, 30, 136–140.

Boja ES, Hoodbhoy T, Garfield M, Fales HM (2005) Structural conservation of mouse and rat zona pellucida glycoproteins. Probing the native rat zona pellucida proteome by mass spectrometry. Biochemistry 44: 1644516460.

Ceko MJ, O'Leary S, Harris HH, Hummitzsch K, Rodgers RJ (2016) Trace Elements in Ovaries: Measurement and Physiology. Biol Reprod.94:86. doi: 10.1095/biolreprod.115.137240.

Juling S, Bachler G, von Götz N, Lichtenstein D, Böhmert L, Niedzwiecka A, Selve S, Braeuning A, Lampen A (2016) In vivo distribution of nanosilver in the rat: The role of ions and de novo-formed secondary particles. Food Chem Toxicol. 97: 327-335.

Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung YH, Kwon IH, Jeong J, Han BS, Yu IJ (2008) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol. 20: 575–583.

Lankveld DP, Oomen AG, Krystek P, Neigh A, Troost-de Jong A, Noorlander CW, Van Eijkeren JC, Geertsma RE, De Jong WH (2010) The kinetics of the tissue distribution of silver nanoparticles of different sizes. Biomaterials 31: 8350-8361.

Lansdown AB (2007) Critical observations on the neurotoxicity of silver. Critical Reviews in Toxicology 37: 237– 250.

Lansdown ABG (2010) A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. Adv Pharmacol Sci. 2010, ID 910686.

Loeschner K, Hadrup N, Qvortrup K, Larsen A, Gao X, Vogel U, Mortensen A, Lam HR, Larsen EH (2011) Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part Fibre Toxicol. 8: 18.

Pang C, Brunelli A, Zhu C, Hristozov D, Liu Y, Semenzin E, Wang W, Tao W, Liang J, Marcomini A, Chen C, Zhao B (2016) Demonstrating approaches to chemically modify the surface of Ag nanoparticles in order to influence their cytotoxicity and biodistribution after single dose acute intravenous administration. Nanotoxicology 10: 129-139.

Rungby J, Danscher G (1983a) Neuronal accumulation of silver in brains of progeny from argyric rats. Acta Neuropathol. 61: 258-262.

Rungby J, Danscher G (1983b) Localization of exogenous silver in brain and spinal cord of silver exposed rats. Acta Neuropathol. 60: 92–98.

Thomson CD, Robinson BA, Stewart RD, Robinson MF (1975) Metabolic studies of [75Se]selenocystine and [75Se]selenomethionine in the rat. Br J Nutr. 34: 501-509.