ASSESSMENT OF SILVER TOXICOKINETIC PARAMETERS: DESKTOP REVIEW AND CRITIQUE OF KEY PUBLISHED DATA

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CONTENTS

INTRODUCTION

This report contains an evaluation of mammalian toxicokinetic (TK) information for silver substances published in peer reviewed journals (up to December 2018) and equivalent data sources, together with commentaries on selected studies. The main objective of the desktop study was to array and interpret key TK data on those Ag substances considered to be representative of ionic and elemental forms (including nanosilver). Further detail on aims is provided in the following sections. In accord with REACH guidance and other guidelines (e.g. WHO IPCS and OECD monographs), emphasis was given to highest tier information from in vivo TK studies, but some reports concerning in chemico speciation and bioelution investigations and TK modelling in silico were included where these were particularly relevant. Unless otherwise stated, Ag nanoforms such as silver nanoparticles (AgNP) were considered to contain silver in the zero oxidation state (Ag^0).

Primary aims of the desktop assessment

(a) The identification and assessment of key TK data on Ag substances which was judged to inform on both systemic and tissue exposure in mammals (with a focus on rodent experimental species, and particularly the rat).

(b) The conduct of a detailed appraisal of such data in its application to total systemic bioavailability estimates, e.g. whether it is adequate to allow the calculation of Ag in blood AUC values for the Ag reference substance(s) in question. Determinations of AUC are of most value in establishing comparative bioavailability between Ag reference substances and may be useful in estimating actual oral absorption values.

(c) An assessment of key TK information pertaining to distribution of Ag substances (particularly following oral administration), and the examination of trends in specific tissue exposure patterns.

(d) The evaluation of the robustness of each designated key study and an examination of its scientific reliability. In addition, an assessment was made of its degree of congruence with other TK data available for the same or similar Ag reference substance(s). Where cases of non-congruence were identified, the basis for the apparent difference was explored, e.g. whether it was due to differences in intrinsic physico-chemical parameters for the Ag substance (particularly in the case of nanosilver forms), or divergences in exposure duration or dosing regimens, or because of other factors.

(e) To establish whether any reliable TK investigation has been published on bulk Ag^0 forms (i.e. micron-size or larger). This has previously been identified as a possible data gap for elemental Ag.

(f) An evaluation of the applicability and reliability of conventional bioelution approaches to the prediction of TK properties for Ag, and in particular to estimations of bioaccessibility/bioavailability.

(g) In overview, this information is intended to be applied for several purposes: (i) to guide the development of an improved grouping and read-across strategy for Ag substances; (ii) to identify data gaps and therefore targets for future TK work programs; (iii) to assist with the interpretation of outcomes of various mammalian toxicity studies on Ag substances, and in particular investigations of reproductive toxicity in rodent species; (iv) as part of the information package to be considered in terms of the optimal design of a proposed new

reproductive toxicity study in the rat, viz. an extended one generation reproductive toxicity study (EOGRTS); and (v) to decide whether non-in vivo approaches such as bioelution or other modelling are viable in the case of Ag–for instance, in the context of substance grouping for mammalian toxicity endpoints.

Subsidiary aims of the desktop assessment

There were also a number of secondary aims to the project:

(a) An appraisal of the designated key studies in respect of other TK parameters of interest: such as information on steady state, accumulation kinetics, clearance, elimination mode as well as other parameters.

(b) For the previously mentioned priorities of systemic exposure/tissue exposure-related TK parameters, a comparison of profiles between various ionic forms of Ag (such as the nitrate and acetate salts).

(c) Conduct of an equivalent comparison between ionic and elemental Ag (the latter encompassing AgNP).

(d) For AgNP, an examination of which primary factors appear to influence the TK profile (including bioavailability), e.g. nanoparticle size, coating/stabilisation system, co-existent ionic silver fraction in the test article etc.

(e) To establish whether any reliable TK data exists for a specific silver ionic compound, viz. AgCl which is of interest due to it featuring in reports of mechanisms involved in the reproductive toxicity of Ag.

(f) To compile a listing of studies which have focussed on TK parameters of relevance to transplacental and embryo-fetal exposure.

Preliminary screening of toxicokinetic reports

Initial bibliographic searches of primary and grey reference sources identified more than 200 individual reports which contained TK information (as either a primary or secondary part of the experimental investigations). These were then graded for relevance using a systematic approach – refer to section ‘Designation of key studies’.

In respect of investigations pertaining to Ag bioelution and speciation studies, account was taken of the existing knowledge and endpoint summaries in the REACH dossiers for silver (refer to the endpoint summary information in the dossiers within the “Toxicokinetics, metabolism and distribution” sections), together with the established outcomes from proprietary bioelution studies commissioned by EPMF (Midander and Wallinder, 2009) and ESTF (O’Connor and Woolley, 2010). Commentary on this work is not duplicated here. The studies critiqued in this report are specifically ones which inform on the validity of bioelution for the purpose of Ag bioavailability prediction, and also on the feasibility of designing further bioelution investigations (see also the section in Conclusions: ‘Feasibility of applying bioelution modelling to elucidate Ag oral bioavailability’).

Designation of key studies

A systemic gating process was applied to the initial set of TK studies of potential interest. This was expert judgement-based, including an assigned relevance of the study dataset to the aims of the desktop review, and an initial assessment of the study robustness and its scientific quality. Those remaining within the gating boundaries after this initial evaluation step were those designated as potentially 'key' or 'supporting' value and hence those requiring a detailed technical assessment and final ranking. Refer to Table 1a-c for a listing of these studies and the final ranking which was applied.

Table 1a

TABLE KEY

A = absorption D = distribution

E = elimination O = ancillary TK parameter

□ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw.

Reference	Title	Ag form	Route	Key study	Supporting study	Not relevant / Excluded	Species	Dosing regimen	Dose-level
Barraclough and Cotton, 2017	Silver nitrate: 28 day oral (gavage) administration toxicity study in the rat; Final toxicokinetic analysis report.	$AgNO_3$	Oral	● A			rat	4-wk	□□
Boudreau et al., 2012	An evaluation of the toxicological effects of discrete sizes of silver nanoscale particles (AgNP) in the Sprague Dawley rat. Presentation / personal communications.	AgAc AgNP (10 nm; 75 nm; 110 nm – citrate capped)	Oral i.v.	● A			rat	Single	□□
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.	AgNP <20 nm; <15 nm (both capped) $AgNO_3$	Oral	● A	● D, E		rat (♂)	4-wk	□ $_{Ag^+}$ □□ $_{NP}$
Park et al., 2011	Bioavailability and toxicokinetics of citrate-coated silver nanoparticles in rats. Arch Pharm Res. 34: 153–158.	AgNP (coated)	Oral i.v.	● A		● D	rat (♂)	Single	□
Boudreau et al., 2016	Differential effects of silver nanoparticles and silver ions on tissue accumulation, distribution, and toxicity in the Sprague Dawley rat following daily oral gavage administration for 13 weeks. Toxicol Sci. 150:131-160.	AgAc AgNP (10 nm; 75 nm; 110 nm – citrate capped)	Oral	● D			rat	13-wk	□□□
Klaassen, 1979	Biliary excretion of silver in the rat, rabbit, and dog. Toxicol Appl Pharmacol. 50: 49-55.	$^{110}AgNO_3$	i.v.	● E	● D		multiple	Single	□
Gregus and Klaassen, 1986	Disposition of metals in rats: a comparative study of fecal, urinary, and biliary excretion and tissue distribution of eighteen metals. Toxicol Appl Pharmacol. 85: 24-38.	$^{110m}AgNO_3$	i.v.	● E	● D		multiple	Single	□

Table 1b

TABLE KEY

A = absorption D = distribution

E = elimination O = ancillary TK parameter

□ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw.

Reference	Title	Ag form	Route	Key study	Supporting study	Not relevant / Excluded	Species	Dosing regimen	Dose-level
Park, 2013	Toxicokinetic differences and toxicities of silver nanoparticles and silver ions in rats after single oral administration. Journal of Toxicology and Environmental Health, Part A, 76: 1246-1260.	Ag/$AgNO_3$ AgNP (8 nm; citrate capped)	Oral		● A		rat (♂)	Single	□ / □□
Bachler et al., 2013	A physiologically based pharmacokinetic model for ionic silver and silver nanoparticles. International Journal of Nanomedicine 8: 3365-3382.	$AgNO_3$; AgAc; other Ag^+ AgNP (various) [Model data]	PBTK		● A, D, E		rat model	Repeat*	□ to □□□
Juling et al., 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.	$AgNO_3$ AgNP (~15 nm; POE capped)	Oral i.v.		● A, D		rat (♂)	Single	□ / □□
Liu et al., 2012	Chemical transformations of nanosilver in biological environments. ACS Nano. 6: 9887–9899.	AgNP (5 nm; ~30 nm; citrate capped)	In chemico		● A, O		N/A	N/A	N/A
Walczak et al., 2013	Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology 7: 1198-1210.	$AgNO_3$ AgNP (60 nm; citrate capped)	Bioelution		● A, O		N/A	N/A	N/A
Loeschner et al., 2011	Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part Fibre Toxicol. 8: 18.	AgAc AgNP (14 nm; PVP capped)	Oral		● D		rat (♀)	4-week	□
Lankveld et al., 2010	The kinetics of the tissue distribution of silver nanoparticles of different sizes. Biomaterials 31: 8350-8361.	AgNP (20; 80; 110 nm)	i.v.		● D		rat	5-day	□
De Jong, 2012	Toxicokinetics and toxicity of nanosilver. [Personal communication; part-published in Lankveld et al., 2010 – see above.]	AgNP (20 nm; 80; 110 nm)	i.v.		● D		rat	5-day	□
Bergin et al., 2016	Effects of particle size and coating on toxicologic parameters, fecal elimination kinetics and tissue distribution of acutely ingested silver nanoparticles in a mouse model. Nanotoxicology 10: 352-360.	AgAc AgNP (20; 110 nm; citrate capped)	Oral		● D, E		mouse (♂)	3-day	□

* Up to 4 weeks duration repeated dosing.

Table 1b (cont.)

TABLE KEY

A = absorption D = distribution

E = elimination O = ancillary TK parameter

□ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw.

Reference	Title	Ag form	Route	Key study	Supporting study	Not relevant / Excluded	Species	Dosing regimen	Dose-level
Furchner et al. 1968	Comparative metabolism of radionuclides in mammals-IV. Retention of silver-110m in the mouse, rat, monkey, and dog. Health Physics 15: 505-514	$^{110m}AgNO_3$	Oral i.v. i.p.		● D, E		multiple	Single	□
Pang et al., 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.	AgNP (multiple capping types) Ag^+	i.v.		● D, O		mouse	Single	□

Table 1c

TABLE KEY

A = absorption D = distribution

E = elimination O = ancillary TK parameter

□ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw.

Reference	Title	Ag form	Route	Key study	Supporting study	Not relevant / Excluded	Species	Dosing regimen	Dose-level
Kim et al., 2008	Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhalation Toxicology 20: 575-583.	AgNP (60 nm)	Oral			Data-pooling*	rat	4-wk	□□ to □□□
Xue et al., 2012	Acute toxic effects and gender-related biokinetics of silver nanoparticles following an intravenous injection in mice. J. Appl. Toxicol. 32: 890–899.	AgNP (15 nm)	i.v.			●	mouse	Single	□□□
Lee et al. 2013	Biopersistence of silver nanoparticles in tissues from Sprague–Dawley rats. Part Fibre Toxicol. 10: 36.	AgNP (10 nm; 25 nm; citrate capped)	Oral			●	rat	4-wk	□□□
Qin et al., 2016	Toxicological evaluation of silver nanoparticles and silver nitrate in rats following 28 days of repeated oral exposure. Environ Toxicol. 32: 609-618.	$AgNO_3$ AgNP (>25-<50 nm; PVP capped)	Oral			●	rat (♂)	4-wk	□

* Considered only as part of a verification dataset with other studies with higher reliability rankings.

After each individual study was subjected to a detailed appraisal it was ranked as either **Key** (Table 1a), **Supporting** (Table 1b) or **Not Relevant/Excluded** (Table 1c).

For further perspective, the test species used in any in vivo work, the study duration and the administered Ag equivalent dose (stratified) is also presented in the tables. Refer to the section covering 'Individual study assessments' for further details on the study experimental procedures, a summary of TK findings, robustness/reliability evaluations, and interpretative commentary on outcomes.

In terms of the nature of the Ag test articles, this assemblage of reports covers two ionic Ag forms ($AgNO_3$ and AgAc), and also a variety of differently sized AgNP (size range: ~5 to 110 nm). Both uncapped (uncoated) and capped (coated) AgNP types are represented within the dataset.

TK parameter definition / Derivation methodology

Definitions and derivations (operational definitions) for TK parameters which are referenced in the individual study assessments and Conclusions section are given below.

C_{max} Maximum plasma concentration; as evaluated by visual inspection of the concentration-time profile.

t_{max} Time of maximum plasma concentration; as evaluated by visual inspection of the concentration-time profile.

Elimination half-life ($t_{½}$), determined by linear regression of at least three data points (not including C_{max}) on the log concentration vs time plot, with a correlation co-efficient (R2) ≥ 0.9.

$AUC_{(0-t)}$ Area under the plasma concentration-time curve from 0 to t, where t is the time of a measurable concentration, calculated by non-compartmental analysis using the log/linear trapezoidal rule.

$AUC_{(0-\infty)}$ Area under the plasma concentration-time curve extrapolated to infinite time, calculated by non-compartmental analysis using the log/linear trapezoidal rule.

Dose normalised values for $AUC_{(0-t)}$ and C_{max} [$AUC_{(0-t)}$/D and C_{max}/D], are estimated by dividing the respective toxicokinetic parameter by the dose level (mg/kg bw).

Dose proportionality values for $AUC_{(0-t)}$ and C_{max} are calculated by dividing the respective toxicokinetic parameter by the corresponding value in the lowest dose group and comparing with the corresponding fold change in any higher dose-group.

Accumulation ratios (RA) based on $AUC_{(0-t)}$ and C_{max} are calculated by dividing the respective value at any specified timepoint after repeated dosing by the corresponding value at an earlier timepoint (conventionally Day 1) of dosing.

Bioavailability (F) is derived from the selected AUC parameter as follows:

$$F = 100\% \times (AUC_{oral} / AUC_{IV}) \times (Dose_{IV} / Dose_{oral})$$

Other remarks related to TK parameters

Oral bioavailability can most reliably be determined from the blood kinetics of a substance administered via both the oral and IV routes as comparators. In considerations of systemic exposure, the bioavailability of a substance is generally preferred over estimates of the percent absorption. Several caveats apply to what constitutes reliable data for this purpose, for instance the situation may become more complex if exposures result in non-linear kinetics, so it is important that assessments determine whether the selected doses resulted in outcomes within the linear range (a detailed discussion of TK principles is not within the scope of this report; readers are instead referred to standard reference texts). See also section '*TK parameter definition / Derivation methodology'* regarding the calculation of oral bioavailability (F).

Where reference is made to any estimate of the fraction of oral dose which is absorbed it is important to recognise that this does not equate to a true measured percent absorption value. In the case of silver where the biliary mode of elimination is the predominant one, the latter parameter would be most properly derived via a specifically designed biliary elimination study[1].

Abbreviations / Glossary

AAS	Atomic absorption spectrometry
AgAc	Silver acetate
$AgNO_3$	Silver nitrate
BBB	Blood brain barrier
BPEI	Branched polyethyleneimine
BTB	Blood testis barrier
CMC	Carboxymethylcellulose
CNS	Central nervous system
d	Day / Study day
DLS	Dynamic light scattering
f	Female(s)
EDX	Energy dispersive X-ray spectroscopy (SEM-EDX)
EOGRTS	Extended one-generation reproductive toxicity study
EPMF	European Precious Metals Federation
ESTF	European Silver Task Force. A sector organisation with interests in biocidal applications of silver.
GD	Gestational day
GI	Gastrointestinal
h	Hour(s)
i.p.	Intraperitoneal
i.v.	Intravenous
LOD	Limit of detection
m	Male(s)
N/A	Not available / derivable

[1] In a biliary elimination study, an oral route of administration is typically used. The bile ducts of at least four animals of the appropriate sex (or of both sexes) are cannulated and a single dose is administered. Sequential measurements of bile are performed for long as necessary to estimate percentage of administered dose which is excreted via the biliary route, which can be used to directly calculate the extent of oral absorption, as follows:

Percent absorption = (amount in bile + urine + expired air + carcass without GI tract contents) / amount administered x 100

n.d. / nd	Not determined or Not detectable
NM	Nanomaterial
NP	Nanoparticle(s)
NR	Not reported
NS; ns	Not stated
OGRTS	One-generation reproductive toxicity study
PEG	Polyethylene glycol
PVP	Polyvinylpyrrolidone
PBPK	Physiologically-based pharmacokinetic (model)
PBTK	Physiologically-based toxicokinetic (model)
SE	Standard error (of the mean)
SEM	Scanning electron microscopy
Soluble form	Used as a shorthand description/grouping for silver forms which have conventionally been considered to have at least moderate water solubility.
TEM	Transmission electron microscopy
TG	Test guideline (e.g. OECD or similar harmonised guideline)
Wk	Week(s)
XRD	X-ray diffraction (XRD)

Notes

(1) Unless otherwise stated, for ease of comparison all in vivo dose-levels referenced in this report have been normalised to equivalent Ag values, e.g. mg Ag/kg bw. In some cases, these have been rounded (with appropriate attention to acceptable precision).

(2) Where dimensions of nm are assigned to nanoparticle/nanoform test articles, these always relate to a reported average diameter (based on experimental determinations made in the study or supplier information). It is important to distinguish between AgNP diameters determined by electron microscopy on particles (and equivalent measurement techniques on isolated particles) from hydrodynamic diameters measured for AgNP in solution; refer to the individual research publications for the definition of which approach was taken.

(3) In respect of nanoparticle dimensions, the terms ‘small’, ‘medium’ and ‘large’ feature in the published literature, though these are not formally defined, e.g. by standards bodies. Within the context of this review, unless further precision is explicitly stated, they are applied to category descriptions of AgNP as follows: *small* ≤ 20 nm; *medium* > 20 nm to ≤ 60 nm; *large* > 60 nm to an upper boundary of circa 100 nm.

CONCLUSIONS

Unless otherwise specified, throughout this section any reference to in vivo TK work relates to investigations using adult rats as the experimental species. Apart from the investigations referenced in the sub-section 'Studies relevant to transplacental and embryo-fetal TK', any findings described in females are for non-pregnant animals unless stated to the contrary.

Availability of TK information on bulk elemental Ag

Bulk elemental Ag is defined here as at least micron-sized material (with negligible Ag nanoform or freely available ionic Ag content). This includes powder forms, and more massive forms of Ag. No reliable TK studies specific to bulk forms of silver in a zero-oxidation state (Ag^0) were identified during the course of this desktop review. It was noted that one rat study[2] exists which found that the uptake and distribution of micron-sized Ag was much less than observed for AgNP, but the mode of administration was via subcutaneous injection which is not a route commonly selected for conventional TK investigations. Studies relating to elemental Ag as a nanoform, i.e. predominantly AgNP, are included in the section 'Individual study assessments'.

Oral absorption: bioavailability/systemic exposure

Conclusions in this sub-section are based on an overview of outcomes from the studies ranked as either 'Key' or 'Supporting' in respect of the absorption phase (see Table 1a and b), viz. Barraclough and Cotton, 2017; Boudreau et al., 2012; van der Zande et al., 2012; Park et al., 2011; Park, 2013; Bachler et al., 2013; and Juling et al., 2016. These represent in vivo TK investigations or else the application of in vivo data within models. Unless otherwise stated, any remarks refer to absorption and bioavailability via the oral route. When comparing the various reports, due note should be taken that single dose studies are unlikely to have resulted in the attainment of steady state conditions. This is a default assumption which has been applied for context to all such experimental investigations.

Only a few TK reports are available on Ag substances which are evaluated as being robust and which also contain data pertinent to derivation of oral bioavailability estimates. This resolves to information on two ionic silver forms – silver acetate (AgAc) and silver nitrate ($AgNO_3$), and to nanoparticulate elemental Ag (AgNP) of various sizes and associated capping systems. As stated in the previous sub-section, no oral bioavailability information was identified for elemental Ag in bulk form (i.e. particulate of at least micron-size range and other massive forms).

Even for Ag substances where information is available, the paucity of TK data does have some impact on the confidence level associated with estimates of oral bioavailability – in part due to the necessity to aggregate data from single and repeated dose investigations and from a quite wide spread of dose levels. It is concluded that data gaps related to absorption phase bioavailability (achieved systemic exposure) currently exist for ionic and elemental Ag, and consideration should be given to progression of further in vivo TK investigations, preferably involving direct comparison of multiple Ag reference substances of interest.

Based on a synthesis of findings from the studies detailed above, it is clear that the absolute bioavailability of Ag (ionic and elemental NP forms) is low, and certainly below 5% based on available data on absorption characteristics and systemic exposure. From investigations which

[2] Tang J et al. (2009) Distribution, translocation and accumulation of silver nanoparticles in rats. J Nanosci Nanotechnol. 9: 4924-4932.

support calculation of oral bioavailability (F), the maximum value evident for ionic Ag did not exceed 4% (Boudreau et al., 2012; and Bachler, 2013).

With respect to derived bioavailability (F values) or estimates of orally absorbed fraction of the administered dose, most of the studies support the conclusion that ionic Ag possesses greater bioavailability via the oral route than is typical for AgNP. The reader is referred to the individual study assessments section in respect of the relative quantitative differences which have been reported (e.g. in Bachler et al., 2013; Boudreau et al., 2012; Park et al., 2013; van der Zande et al., 2012). Achieved tissue concentration datasets provide further evidence that ionic Ag forms have relatively greater systemic availability than AgNP following either single-dose (Park et al., 2013) or repeat-dose (Loeschner et al. 2011; van der Zande et al., 2012) administration of sub-toxic amounts of Ag substances. In terms of contradictory reports, it was noted that a different inference was drawn by Juling et al. (2016) based on a relatively limited scope in vivo study and then application of a PBTK model. These investigators deduced that no difference existed between ionic and NP forms of Ag.

Notwithstanding the apparently lower bioavailability of AgNP, it is important to note that variability does exist in the dataset as a whole, especially for smaller size range AgNP (<15 nm). Appreciable bioavailability was evident for 10 nm AgNP (Boudreau et al., 2012) and 8 nm AgNP (Park et al. 2011) in single dose experiments. Whereby F values of 3.5% / 3.2% (m/f) were derived from Boudreau et al., and 1.2-4.2% from Park et al., 2011 (in the latter investigation, F values in the single-sex design showed a dose-dependency for male animals).

The overall dataset broadly supports a conclusion that larger AgNP have relatively lower bioavailability; for instance, compare the F values obtained for AgNP-110 nm with those for AgNP-10 nm in Boudreau et al., 2012. Occasional conflicting outcomes in relation to the influence of size have been reported, e.g. Bachler and co-workers (2013) derived equivalently low bioavailability estimates (of circa 0.9%) via a PBTK model for both a small- and a medium-sized AgNP (14 nm and 60 nm). Given that inter-study comparisons are complicated by experimental diversity (duration, dose level etc.), and because multi-factorial influences intrinsic to the AgNP test articles may be operating (co-existing ionic Ag content, capping system differences etc.), some caution is appropriate in not over-interpreting the currently available dataset. Refer also to further remarks in the sub-section 'Influence of nanoparticle size as a factor in AgNP TK'. As discussed therein, there is rationale for why exposure to smaller AgNP would be expected to result in greater systemic exposure; and on balance the weight of evidence is supportive of greater bioavailability for such AgNP.

Even allowing for the variation in study findings, if AgNP is used as a reference substance for elemental Ag in toto and conservative assumptions are applied, the existing data do not support a conclusion of negligible bioavailability via the oral route. This has important implications if Ag nanoforms are selected by default as a read-across substance to other forms of elemental Ag.

For the only two ionic Ag forms with sufficient data available, viz. $AgNO_3$ and AgAc, their respective bioavailability via the oral route appears to be of the same order (based on results following a single dose administration), but appears to be somewhat greater for AgAc. Calculated $AUC_{(0\text{-}t)}$ / D results from Barraclough and Cotton (2017) and Boudreau et al. (2012) are presented in Table 2, since these two studies are sufficiently similar in their experimental design to facilitate a direct comparison. It may be inferred that, dependent on gender, the achieved systemic exposure was 25-100% greater in the case of AgAc. This therefore supports the selection of AgAc as a 'worst-case' test article where read-across to ionic Ag forms is contemplated.

	$AUC_{(0-t)}$ / D	
	♂	♀
$AgNO_3$ [Barraclough and Cotton (2017)]	227	311
AgAc [Boudreau et al. 2012]	283	623

Table 2. Comparative systemic exposure data for silver nitrate ($AgNO_3$) and silver acetate (AgAc). Data are derived from d1 sampling. Closest dose level matched as a point of departure prior to derivation of $AUC_{(0-t)}$ / D values (refer also to individual study summaries).

Most data relate to rodent species. However, from a series of dated but comprehensive studies of blood, plasma and biliary levels (Klaassen, 1979; Gregus and Klaassen, 1986) using $AgNO_3$ as an ionic Ag reference, it can be securely assumed that for a given dose of ionic Ag the achieved systemic exposure will be higher in the rat as a test species rather than other typical non-rodent laboratory animal species (rabbit, dog, and non-human primates).

Gender differences in TK

Where animals from both sexes were investigated, data from several studies on ionic Ag and AgNP indicates that moderately greater systemic availability was observed for females (see also the study summaries for Barraclough and Cotton, 2017; Boudreau et al., 2012[3]; and Kim et al., 2008). For example, from the d1 and terminal blood sampling data it was noted that consistently higher $AUC_{(0-t)}$ values were evident in a 4-week oral study on $AgNO_3$ (Barraclough and Cotton, 2017) thereby resulting in circa 10-80% differences in female versus male groups at equivalent dose levels. In the study by Boudreau and co-workers, mean C_{max} and $AUC_{(0-t)}$ values were greater in the case of females, both for an ionic Ag form (AgAc) and for AgNP.

Although the study by Xue et al. (2012) had various design and robustness limitations and was not ranked as 'Key' or 'Supporting', some gender differences were also evident in mice administered a single high i.v. bolus dose of a small AgNP (15 nm). Group mean $AUC_{(0-\infty)}$ for females was about 30% higher than in males; the elimination half-life in female mice ($t_{1/2}$ = ~30h) was more protracted (being approximately double that observed in males); and Ag concentrations in the spleen were relatively greater in females, although levels were comparable in the other organs examined.

The weight of evidence is that for equal administered doses, achieved systemic exposure tends to be slightly to moderately greater in females than in males for ionic Ag and AgNP. It should be borne in mind that a number of the studies reviewed in this document cannot directly inform on gender differences as they were of single-sex design.

Steady state conditions

Information on systemic exposure steady state following repeated oral dosing is available from the studies conducted by Barraclough and Cotton (2017) on $AgNO_3$; and from van der Zande et al. (2012) on $AgNO_3$ and AgNP (small capped and uncapped NP types). The ionic Ag dose levels in the two studies were 13-64 mg Ag/kg bw/d, and 6 mg Ag/kg bw/d, respectively. A common finding from the Ag in blood determinations was that, in the case of both the ionic Ag and AgNP test articles, steady state kinetics had apparently been achieved by the end of 4

[3] As an exception in this study, the oral bioavailability value only for small 10 nm AgNP was minimally greater in males than females.

weeks of dosing. The serial Ag in blood measurements performed by van der Zande and co-workers allow for more precision on the time-course, with steady state conditions being evident after 14 days of dosing. This temporal estimate of time to steady state is also supported by extrapolations made using elimination half-life (refer to next sub-section). Some insights on individual tissue kinetics can be gleaned from the serial assessments conducted by Lankveld et al. (2010) after treatment of rats with several types of AgNP, but this only covered a 5-day dosing period which does not assure achievement of steady state.

Elimination half-life / Clearance (systemic)

Based on several reports which evaluated Ag in blood concentrations, elimination half-life ($t_{1/2}$) estimates in rats for ionic Ag and AgNP range from 24 h to 30 h. It should be noted that in the case of AgNP these estimates are derived from digestion methodologies which resolve Ag content as the ionic form. In a repeat-dose rat study using $AgNO_3$ (Barraclough and Cotton, 2017) it was reported that $t_{1/2}$ values could not be accurately derived for the dosed groups with the exception of the mid-dose (32 mg Ag/kg bw/day) males at the Wk-4 timepoint. However, this single derived value was consistent with other reports (at circa 30 h). One non-congruent report (Lee et al., 2013) estimated much longer $t_{1/2}$ values in rats for citrate-capped AgNP-10nm and Ag-NP-25 nm, in the region of 78–140 days, but it is considered not to be a reliable study. A single-dose study using the i.v. route conducted by Pang et al. (2016) calculated a broad range of elimination half-life values for AgNP with different capping systems, but that study is also considered to be of lower reliability in respect of this TK parameter.

Several studies provide useful information on systemic clearance of Ag following oral or parenteral route administration. These include reports by Lankveld et al., 2010; Park et al., 2011; Boudreau et al., 2012; van der Zande et al., 2012; Bachler et al., 2013; and Pang et al., 2016 (refer to the individual study summaries for detailed information). Although some variation in clearance rates was evident, a common finding was rapid systemic clearance irrespective of the administered dose and the form of Ag (ionic and NP).

Distribution of Ag to tissues

Tissue distribution patterns and related biokinetics represent the most commonly studied TK parameters for all Ag forms and comprise the greatest number of individual publications (refer to Table 1/individual study summaries). Most of the work in this area is based on findings from a terminal sacrifice at the end of the dosing period. It should be noted that there are variations in detailed experimental design in distributional studies; in particular, not all studies incorporated an extensive set of tissues. Unless stated to the contrary, conclusions in this sub-section are based on the parameter of Ag concentrations per unit weight of sampled tissue.

A very common observation is the preferential distribution of Ag to the organs of the reticulo-endothelial system (liver and spleen) irrespective of the route of administration, and whether the administered form was ionic Ag or AgNP. This is the case in multiple species including rodents (rats and mice), rabbits, dogs and non-human primates (see for example, Klaassen, 1979). As might be expected, another consistent finding is the detection of high amounts of Ag associated with various gut regions in the case of oral route studies. This distribution pattern is also consistent across a wide range of administered doses, and for single and repeat dose exposures. Along with the blood, the <u>total</u> Ag depots associated with these tissues tend to be considerable compared to other most other distribution sites.

A number of studies have identified several other tissues where there is moderate distribution of Ag, viz. the lungs, kidneys, heart, pancreas and lymph nodes (though the dataset is quite

small for the last three tissues). The balance of evidence is that accumulation of Ag in the kidneys is more often associated with smaller AgNP (e.g. see Loeschner et al., 2011 and van der Zande et al., 2012). For some types of AgNP, the relatively high lung Ag concentrations may be attributable to NP charge state (Pang et al., 2016). Contradictory findings in the qualitative patterns of distribution have been described for both ionic Ag and AgNP as to whether substantive distribution to the CNS (brain) and testis occurs. These controversial circumstances are discussed in subsequent sub-sections ['Distribution to brain/CNS' and 'Distribution to reproductive organs (testis / ovaries / uterus)'].

Another finding replicated in many of the studies is that dose-normalised tissue concentrations are typically higher for ionic Ag versus AgNP. This is to be expected based on the relatively higher bioavailability of the ionic form.

Given considerations around assured steady state conditions, data from repeated exposure investigations in vivo (possibly supported by PBTK models) are expected to be most relevant to the interpretation and design of repeat dose mammalian toxicity studies, including EOGRTS/OGRTS. TK studies of this duration which are also fully robust are comparatively few in number, and none of these investigate Ag concentrations across the full set of reproductive tract tissues which are of interest (including the testis, ovary and uterus, as well as accessory organs). Hence the datasets need to be aggregated to provide a more comprehensive picture. The most useful studies of 4 weeks duration or longer are evaluated as those published by Loeschner et al., 2011, van der Zande et al., 2012 and Boudreau et al., 2016; with work by Kim et al. (2008) also having some utility[4] in a supporting role. Information from single dose work, or else repeated dose experiments of a few days duration such as Lankveld et al., 2010 and Bergin et al. 2016 (using mice), can provide additional insights. Only the 13-week rat study performed on AgAc (and 3 types of AgNP) by Boudreau et al. (2016) covers a treatment period exceeding 4 weeks, and therefore it represents one which has specific relevance to the duration of the default pre-mating treatment period in EOGRTS/OGRTS. The qualitative distribution pattern for the low dose group in this study (65 mg Ag/kg bw/d) is likely to be informative in respect of TK for the high-dose group in the OGRTS reported by Sprando et al (2017). The full dataset has been abstracted from the publication and is detailed in Appendix 1 of this report. In terms of the coverage of reproductive tract tissues, data is only available for the uterus.

Several methodological and interpretative caveats apply to Ag distribution datasets:

(a) When considering quantitative distribution of Ag within the body to judge the deposited dose, it is important to also normalise Ag concentration data expressed in amount per unit weight of the individual tissue or organ to account for total tissue/organ mass (and therefore gain an appreciation of the total amount of Ag located in any specific compartment). This becomes more significant in the case of the largest organs such as the liver, or the instance of total blood volume considered as a tissue.
(b) Highly vascularised organs will have a comparatively larger fraction of their Ag depot associated with that vascular compartment.
(c) Without direct localisation evidence, it should not be assumed that all Ag in tissues is intra-organ located since the possibility exists that there may be a substantial fraction associated with the endothelial sub-compartment (given that Ag binds to endothelial epithelial cell membranes).
(d) The most common measurement techniques based on acid digestion and ICP-MS (or alternate spectroscopic detection) cannot speciate the form of Ag present. Visualisation via TEM/SEM, with or without EDX or autometallography, or else single particle-ICP-MS can provide more precision in this regard.

[4] This study contains terminal Ag tissue distribution data, but there are reservations about the overall study robustness and relevance.

(e) Finally, it should be noted that not all Ag will necessarily be recovered by the commonly used techniques for its dissolution and extraction. Many strong-acid tissue digestion methods will underestimate the absolute amount Ag present as certain highly insoluble forms; for example, nitric acid digestion alone may not completely solubilise AgCl or AgCl-complexes, and the degree of dissolution of AgSe and AgS tissue depots is not well investigated to date (but it is predicted to be incomplete).

Distribution to reproductive organs (testis / ovaries / uterus)

Most data have been generated from TK studies performed in rats and mice. The available information in other species is sparse. Little reliable TK information was identified which was specific to the male accessory sex organs (seminal vesicles, ventral prostate and epididymides) and the uterus in females.

For the testis, individual study datasets related to Ag distribution are conflicting, and related conclusions as to the extent of exposure for this tissue compartment are therefore non-congruent. The following commentary focuses on a number of studies which exemplify the contradictory findings.

A well-conducted 4-week oral study in rats with ionic Ag ($AgNO_3$) and two types of small AgNP (<20 nm; both stabilised, but by different systems) which was performed by van der Zande et al. (2012) ostensibly demonstrated high testicular Ag levels at termination. Dependent on the test article, these either approached or were equivalent to the levels measured in the liver and spleen. No significant difference was evident in the distribution profiles of the two types of AgNPs. The testicular Ag depots were slow to clear when sequentially evaluated for up to 8 weeks post-dosing. In another 4-week oral route study in the rat (Kim et al., 2008)[5] with medium-size AgNP (60 nm; uncapped) administered at high dose levels, the terminal sacrifice testicular Ag concentrations were increased in a dose-dependent manner. Ag levels in the testis were relatively lower when ratioed to liver and blood values than those found by van der Zande and co-workers but were still appreciable in absolute terms. The same group replicated these findings in a longer duration 13-week study[6]. Several reports in mice and also rabbits exist of Ag uptake by the testis after i.v. administration of AgNP. However, these studies are either evaluated as having robustness limitations or else they did not incorporate conventional TK designs adequate to make them useful as comparators[7].

Set against the findings of appreciable distribution Ag to the testis are some contradictory studies. One is a report from Lankveld et al. (2010) on three types of AgNP (20 / 80 / 110 nm) administered on 5 consecutive days to rats (at a dose approximating to 0.1 mg Ag/kg bw/d). Absolute Ag concentrations associated with the testis were low in the case of all test articles, and there was evidence of rapid clearance from the organ. In accord with these findings, only low concentrations of Ag were associated with the testis in rats which received small AgNP (10 nm) at 0.01 or 0.1 mg Ag/kg bw/d for 4 weeks[8]. The mean residence time of the Ag depots in the testis was not protracted and was similar to the main organs of distribution (viz. liver and

[5] This study contains terminal Ag tissue distribution data, but there are reservations about the overall study robustness and relevance.

[6] Kim YS et al. (2010) Subchronic oral toxicity of silver nanoparticles. Particle and Fibre Toxicology 7:20.

[7] Lee et al. (2013) which is separately evaluated in this report / Garcia TX et al. (2014) Sub-acute intravenous administration of silver nanoparticles in male mice alters Leydig cell function and testosterone levels. Reprod. Toxicol. 45: 59–70 / Castelini C et al. (2014) Long-term effects of silver nanoparticles on reproductive activity of rabbit buck. Syst. Biol. Reprod. Med. 60: 143–150.

[8] Lee JH (2018) Tissue distribution of gold and silver after subacute intravenous injection of co-administered gold and silver nanoparticles of similar sizes. Arch Toxicol. 2018 Apr;92(4):1393-1405.

spleen). Very low levels of Ag associated with the testis were also found in a single dose i.v. and oral route study in rats at higher treatment levels with ionic silver ($AgNO_3$) and small AgNP (15 nm) as test articles (Juling et al., 2016).

It is currently unclear why such opposing findings exist, but there are several possible explanations. At least a partial explanation may be the substantial dose level differences spanning the reports. In the specific case of AgNP, some evidence points to size-dependency effect whereby smaller more bioavailable AgNP may be disproportionately distributed to the testis. For instance, when mice were orally administered AgNPs with a range of diameters (22 / 42 / 71 nm) and one sub-micron Ag form (323 nm) at 1 mg/kg/day for 14 days[9], Ag was detected in the testis in the case of 22 nm and 42 nm AgNPs but not after administration of 71 nm or 323 nm particles. It is unclear whether this was simply due to the higher systemic bioavailability predictable for the smaller AgNP, or because of enhanced penetration of the blood-testis barrier, or else multiple factors. As is discussed elsewhere in this review, contaminating ionic fractions in AgNP test articles can complicate straightforward deductions for such nanoparticles.

In the testis, a blood-testis barrier (BTB) divides the epithelia into basal and adluminal compartments whereby the critical germ cells are isolated from the circulatory system. Specialised tight junctions, formed between adjacent Sertoli cells, create a barrier that restricts passage of substances and ions from the systemic circulation (and transfer from the lymphatic system). The BTB can prevent exposure of the germinal epithelium to toxic substances, including metals, though it is known that metal transporter systems exist in the testis which can circumvent this system. Hence, a major consideration is around whether Ag-associated depots are within the testicular germinal epithelial compartment itself, or else are associated with interstitial and endothelial compartments. The use of conventional TEM studies alone to probe this localisation is inherently flawed due to the potential for experimental artefacts, and therefore some published reports should be regarded with caution[10]. One study in rats[11] exists which did examine intratesticular Ag distribution in rats after a single i.v. dose of radiolabelled $^{110m}AgNO_3$ (2 µg Ag/kg bw). Levels of Ag in the testes and epididymes were detectable but low compared to the liver (circa 5% ratioed to the liver). Quantitative localisation metrics were not part of the design, but based on histological visualisation the Ag deposits appeared to be more abundant in interstitial macrophages and the basement membrane, though they were evident to some extent in spermatogenic cell types of all stages (including the acrosome in mature spermatozoa). Silver tends to be preferentially localised to basal lamina in a number of organs, and the observations in this work related to tubular basal lamina are therefore consistent. However, some penetration of the BTB apparently also occurred. High concentrations of metallothioneins (cysteine-rich metal-binding proteins) localised to the membrane of the Golgi apparatus act to protect cells from over-exposure to various essential and non-essential metals (such as copper, selenium, zinc, cadmium and, mercury)[12]. Silver is known to have high avidity for thiol groups, and given these are present in metallothionein cysteine residues, this represents a potential molecular binding target. As has been demonstrated for cadmium, there may be a critical threshold for metal binding to metallothionein, which if exceeded then results in cytotoxicity. The aforementioned areas of intratesticular localisation and molecular targeting

[9] Park EJ et al. (2010) Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. Environ Toxicol Pharmacol. 30: 162-168.

[10] For instance: Castelini C et al. (2014) Long-term effects of silver nanoparticles on reproductive activity of rabbit buck. Syst. Biol. Reprod. Med. 60: 143–150.

[11] Ernst E et al. (1991) Ultrastructural localization of silver in rat testis and organ distribution of radioactive silver in the rat. J Appl Toxicol. 11: 317-321.

[12] Wong EW et al., (2010) Cell junctions in the testis as targets for toxicants, in Reproductive and Endocrine Toxicology (Hoyer PB, Richburg JH, editors. ed) pp 167–188, Academic Press/Elsevier, Oxford.

have not been extensively studied to date, and these remain as research needs in terms of Ag deposition patterns to critical cell types and potential linkages with reproductive toxicity.

To date, a specifically designed testicular biokinetics study comparing the main reference forms of interest for Ag is still missing. Context should also be applied as to whether sufficient evidence exists to link TK outcomes to evidence for adverse testicular effects. Although it was a toxicity study and not a TK report, and is therefore outside the scope of this review, it should be mentioned that a sub-chronic rodent study (aligned with TG 408) including full male reproductive system parameters has been conducted on a small PVP capped AgNP[13]. At quite high dose levels (up to 200 mg Ag/kg bw/d), only minimal treatment-related effects on testis histopathology and sperm parameters were demonstrated.

Limited work has been performed on Ag distribution to the ovaries, and the differing designs of the available investigations restrict what conclusions can be drawn. However, it does not appear that the ovary is a major site of distribution in either rats or mice following the administration of ionic Ag or AgNP in either single or repeat-dose studies (Austin et al., 2012; Xue et al., 2012). A more significant degree of Ag accumulation in the uterus of rat dams was reported by Charehsaz et al. (2016)[14] in a developmental study when AgNP-55 nm (0.2 to 20 mg Ag/kg bw/d) and ionic Ag ($AgNO_3$; 20 mg Ag/kg/day) were dosed orally during gestation days (GD) 7−20. Accumulation of Ag in the offspring was also detected confirming that Ag was able to cross the placenta. Appreciable Ag levels in the uterus were also detected in pregnant mice following i.v. administration of $AgNO_3$ and a small AgNP (10 nm) during GD 7-9[15]. Results from Boudreau et al. (2016) in relation to the uterus after 13 weeks of treatment with AgAc or AgNP (10 / 75 / 110 nm) suggest that the pattern of deposition may differ between larger and smaller AgNP, with the measured Ag concentrations increasing with NP size. However, this is a single report which needs replication. Uterine accumulation of Ag in animals treated with AgAc for 13 weeks was appreciable, e.g. for the group that received 65 mg ionic Ag/kg bw/d. Taken together with outcomes from Charehsaz et al., 2016, this finding is relevant to the interpretation of reproductive toxicity studies in rodents.

Distribution to brain/CNS

Some reviews of Ag TK have concluded that the balance of evidence is that Ag does not extensively transfer into the CNS (brain); in particular, the reader is referred to Lansdown (2007)[16]. However, this remains a matter of ongoing controversy, with conflicting reports for ionic and nanoparticulate forms of Ag and its degree of distribution to the CNS. These studies are of varying durations and use both oral and parenteral administration routes. Some pertinent reports are summarised in Table 3. Studies marked in bold are those considered to be the most central to the nature of the divided evidence. Several of these reports are evaluated in detail in the section covering individual study assessments. Refer in particular to van der Zande et al. (2012), Loeschner et al. (2011), Lankveld et al. (2010), Kim et al. (2008), Pang et al. (2016) and Furchner et al., (1968).

[13] Lafuente D et al. (2016) as previously reviewed in an EPMF report: *Ag literature follow-up project – Appraisal of key references (Medium priority).*

[14] Charehsaz M et al. (2016) as previously reviewed in an EPMF report: *Ag literature follow-up project – Appraisal of key references (High priority).*

[15] Austin et al. (2012) Distribution of silver nanoparticles in pregnant mice and developing embryos. Nanotoxicology 6: 912-922.

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

From Table 3 it may be seen that the preponderance of evidence suggests that the brain is not a major site of distribution in rodent species after administration of Ag via oral or parenteral routes of exposure. In opposition to this supposition, an apparently robust study by van der Zande et al. demonstrated that the brain was a significant site of distribution in rats orally administered ionic Ag ($AgNO_3$) and two types of small AgNP (<20 nm) for 4 weeks. Though Ag concentrations in the brain were highest for ionic Ag, they were still appreciable for AgNP and did not appear to be attributable to ionic Ag contaminant in the test articles nor to capping system influence. Tissue clearance of Ag from the brain occurred at a slow rate with substantial

Studies reporting appreciable distribution into the brain/CNS				**Studies reporting limited distribution into the brain/CNS**			
Reference	***Ag form***	***Design***	***Approach***	***Reference***	***Ag form***	***Design***	***Approach***
Rungby and Danscher, 1983a	Ionic	RD ip	Qualitative {a}	Furchner et al., 1968	Ionic	SD ip	Quantitative
Rungby and Danscher, 1983b	Ionic	RD oral	Qualitative {b}	Shinogi and Maeizumi, 1993	Ionic	SD ip	Quantitative Rats / mice
Stoltenberg et al., 1994	Ionic	SD iv	Qualitative	Kim et al., 2008	NP [m]	RD oral	Quantitative
van der Zande et al., 2012	Ionic / NP [s]	RD oral	Quantitative	Kim et al., 2010	NP [m]	RD oral	Quantitative
Garza-Ocanas et al., 2010	NC [s]	SD ip	Quantitative {c}	**Loeschner et al., 2011**	Ionic / NP [s]	RD oral	Quantitative
				Lankveld et al., 2010	NP [s], [m], [l]	RD iv	Quantitative
				Charehsaz et al., 2016	Ionic / NP [m]	RD oral	Quantitative {d}
				Pang et al., 2016	Ionic / NP [m]*	SD iv	Quantitative Mice
				Lee et al., 2018	**NP [s]**	**RD iv**	**Quantitative**

Table 3. Selected TK studies which contain information related to Ag distribution to the brain. Unless otherwise notated the experimental species used was the rat. Data from inhalation route or intranasal studies is not included (due to dosimetry differences and considerations related to potential for direct transfer of the test article via the olfactory nerve into the CNS).
RD = repeat dose. SD = single dose. ip = intraperitoneal. iv = intravenous. Size of tested Ag nanoform: [s] = small / [m] = medium / [l] = large. NC = nanocrystal. * = AgNP with several capping systems were investigated. {a} = evaluation of brain tissue from offspring after developmental stage exposure to Ag lactate. {b} = oral administration of either Ag lactate or $AgNO_3$. {c} = mixed findings were evident whereby brain Ag levels were relatively high at 24 h but not at 96 h. {d} = pregnant dams exposed on GD 7-20.
References in the Table that are not cited or evaluated elsewhere in this review document:

Rungby J and Danscher G (1983a) Neuronal accumulation of silver in brains of progeny from argyric rats. Acta Neuropathol (Berl) 61: 258-262.
Stoltenberg M et al. (1994) Autometallographic detection of silver in hypothalamic neurons of rats exposed to silver nitrate. J Appl Toxicol. 14: 275-280.
Garza-Ocanas L et al. (2010) Biodistribution and long-term fate of silver nanoparticles functionalized with bovine serum albumin in rats. Metallomics 2: 204-210.
Shinogi M and Maeizumi S (1993) Effect of preinduction of metallothionein on tissue distribution of silver and hepatic lipid peroxidation. Bio. Pharm. Bullet. 16: 372-374.

concentrations still detectable 2 months after cessation of treatment (>90% of those measured at termination of dosing). The study authors point out the disparity in the brain levels versus other published reports but do not offer a definitive explanation for the difference. In weight of evidence terms, this study needs to be placed in the context of several contradictory reports. A study of similar design involving oral exposure of rats to ionic Ag (AgAc) and small AgNP (Loeschner et al., 2011) found that amounts of Ag distributed to the brain were minimal. This was also the case in two well-conducted investigations following i.v. administration of AgNP to rats. Lee et al. (2018) reported that the brain was not a significant site of distribution (e.g. at only ~0.3% of liver Ag levels at both dose levels) in animals which received 0.01 or 0.1 mg Ag/kg bw/d of a small AgNP (10 nm) for a 4-week period. Similar findings were evident in a study by Lankveld et al., 2010 where absolute Ag concentrations in the brain were amongst the lowest in the range of tissues examined after administration of three types of AgNP of varying sizes – circa 1% or less of the administered dose was associated with the brain. A 4-week oral route study in rats (Kim et al., 2008) with a medium-sized AgNP also found unremarkable brain accumulation (being only 1.25 to 2.5-fold higher than blood levels, dependent on dose). Though a 90-d rat toxicity study on AgNP (25 nm) with a subsidiary TK segment[17] found that relatively high levels of Ag were present in the brain of treated animals at termination, the quantitative pattern of distribution in respect of the reticulo-endothelial organs and some other tissues was non-congruent with the overall dataset on Ag – this study should therefore be set aside on scientific reliability grounds.

An obvious explanation for the contradictory findings between the van der Zande et al. report versus most other robust studies is currently lacking, but it is not considered likely that this is due to either methodological variance (e.g. relating to analytical technique or exposure differences), or to parameters intrinsic to the test article (e.g. AgNP size, capping system or contaminating ionic Ag). The brain is highly vascularised, but calculations suggest that the contribution of Ag in the associated blood compartment is not a major consideration, and the organ harvesting techniques in the studies were in any event comparable.

In terms of whether Ag actually penetrates the blood brain barrier (BBB), a Danish research group (1983a[18]; 1983b[19]; 1994[20]) have described the detection of Ag deposits by autometallography within neurones and glial cells in Ag-treated adult rats and neonates which were exposed during development. These publications also make it clear that much Ag is located external to the BBB in the endothelial compartment (cerebral blood vessels), choroid plexus and meninges. In terms of biological plausibility, there are puzzling findings in these reports in respect of the heterogeneity of the Ag distribution within the brain (which was ascribed by the authors to either variation in turnover of neuronal sub-populations, or to regional differences in the permeability of the BBB). Furthermore, there is no obvious explanation of why exposure of adult rats to $AgNO_3$ resulted in more Ag content in glial compartments whereas deposition occurred preferentially in neurones following Ag lactate treatment. Although the localisation findings by this group have been linked to neurotoxicity concerns (an area outside of the scope of this review), the experimental evidence presented by them is not particularly robust in that neurotoxicity and neurobehavioural assessments were not TG-conform. The data are again contradictory as to whether functional effects are linked to Ag deposition in the CNS:

[17] Garcia T et al. (2016) Oral subchronic exposure to silver nanoparticles in rats. Food Chem Toxicol. 92: 177-187.

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

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

[20] Stoltenberg M et al. (1994) Autometallographic detection of silver in hypothalamic neurons of rats exposed to silver nitrate. J Appl Toxicol. 1994 14: 275-80.

Ag treated mice were evaluated as being hypoactive (Rungby and Danscher, 1984[21]), which contrasts with a study in rats where silver deposition in the brain was not associated with any behavioural changes (Stoltenberg et al., 1994).

The available information on transplacental transfer to the CNS of developing animals is also afflicted by disparities. Soluble silver when parenterally administered to pregnant rats has been reported to lead to some deposition in the neurons and glial cells of the brain of offspring (Rungby and Danscher, 1983a), though associated pathological change or CNS dysfunction was not reported. Transplacental toxicokinetics of silver nanoparticles and an ionic silver substance showed that the developing rat brain is not a principal site of silver distribution, and pup brain silver levels after repeat dosing of dams with up to 20 mg Ag/kg bw/d of either test article were only about twice that of controls (Charehsaz et al., 2016). Refer also to the sub-section 'Studies relevant to transplacental and embryo-fetal TK'.

Versus the oral route, other administration modes can have a significant influence on distribution of toxicants to the CNS, for instance in the case of intranasal instillation or inhalation exposure (where direct access via the olfactory nerve is possible). Investigations based on such exposure routes have not been considered here. As a final cautionary point, findings from parenteral administration based on high dose levels/dose rates also need to be considered cautiously in case this artefactually perturbs CNS biokinetics due to the impact of bolus blood concentrations.

Elimination mode (excretion)

Foundation studies in a variety of experimental species (Klaassen, 1979; Gregus and Klaassen, 1986), based on examination of the biliary elimination characteristic for Ag, have demonstrated that this is the primary mode of elimination for systemically absorbed Ag[22]. This conclusion is supported by a number of experiments which have measured differential faecal and urinary Ag content after either ionic Ag or AgNP administration (e.g. Furchner et al., 1968; Park et al., 2011; van der Zande et al., 2012; Bergin et al., 2016). It is apparent that no more than around 1% is eliminated by the renal route. Where Ag was administered via the i.v. route, comparison of the biliary and faecal excretion patterns permits prediction of whether it is eliminated or reabsorbed by the intestine – in the case of Ag the results are strongly supportive of biliary excretion.

The measured rate of biliary elimination shows marked species variation, with the highest rate evident for rats: in relative terms, rat = 1; rabbit = 0.1 and dog = 0.01, with this order correlating to the achieved plasma concentrations of Ag (Klaassen, 1979).

Metals are known to be cleared from the body via two main routes: urinary and fecal elimination. In a systematic comparative study of a total of 18 non-ferrous and ferrous metals, Gregus and Klaassen (1986) delineated the mode applicable in the case of each metal. Biliary elimination was proportionately greater for Ag than any other metal even within the group of metals where this was the predominant mode (i.e. in order of their excretion rates: silver > manganese >> copper > thallium > lead > zinc > cadmium > iron > methyl mercury). This grouped ranking should be borne in mind when inter-metal comparisons involving silver are considered.

[21] Rungby J and Danscher, G (1984) Hypoactivity in silver exposed mice. Acta Pharmacol. Toxicol. 55: 398-401. It should be noted that a conventional neurobehavioural functional battery was not employed in these investigations.

[22] A finding further replicated in other studies, e.g. Tichý P et al. (1986) Biliary excretion of ^{110m}Ag and its kinetics in the isolated perfused liver in rats. J Hyg Epidemiol Microbiol Immunol. 30: 145-148.

Influence of nanoparticle size in AgNP TK

Some commentary on studies demonstrating a relatively higher bioavailability of smaller AgNP (<15 nm) has been provided under a previous sub-section (refer to 'Oral absorption and bioavailability/systemic exposure'). For small AgNP, with correspondingly high surface area to volume ratios, this is not an unexpected finding if it is linked to the consequential influence from biodissolution processes then resulting in the formation of the more bioavailable ionic Ag^+ form. A number of reports[23] on various types of inorganic NP have illustrated that the dissolution of such small NP is correspondingly greater. Given the known surface-active oxidative dissolution of Ag, the effect for small AgNP will be particularly magnified, and this conclusion is corroborated by several chemistry studies[24], as well as the speciation work by Liu and co-workers (2012) which is discussed elsewhere in this review document.

In one of the few robust TK studies comparing a range of AgNP sizes, Boudreau et al. (2012) showed the degree of systemic exposure after single oral or i.v. doses correlated in a monotonic manner with decreasing size for AgNP with diameters of 10, 75 or 110 nm. Another i.v. route study on 20, 80 or 110 nm AgNP (Lankveld, 2010) did show some size-dependent tissue distribution effects, but these were moderate in magnitude and the relationships of blood compartment kinetics with size differed from those in the Boudreau et al. study.

In general terms the relationship of biokinetics with NP size is known to be a complex and multi-factorial one[25]. For instance, if a particular size of NP is more prone to the formation of large agglomerates (e.g. due to charge state), it is predicted that the increased scavenging by the reticulo-endothelial system will result. Such agglomeration can also be driven by factors other than size. As a further variable, the degree of protein opsonisation (corona formation around NP) can also be influential. In addition, both endothelial barrier transit and also clearance processes are likely to differ for very small NP. In the latter case due to increased potential for renal elimination (with higher kidney levels being an associated event).

As a final remark: though it remains to be experimentally verified, it is predicted that the TK behaviour of micron-sized Ag powder is likely to differ from AgNP. This is based on extrapolations of the predicted surface-area effects and their impact on Ag^+ formation in vivo.

Influence of AgNP coating/capping system[26] on TK

Capping agents (small organic ligands, polymers, surfactants, etc.) are often utilised in the synthesis of AgNP of controlled size and defined shape, and then to disperse and stabilise the NP in suspension (countering their tendency to agglomerate due to charge state). Stabilisation systems which have been applied for AgNP commonly include citrate, polyvinylpyrrolidone (PVP), polyethyleneglycol (PEG), branched polyethyleneimine (BPEI) and polyvinyl alcohol (PVA); as well as others, e.g. polysaccharides, carbon, hydrocarbons, starch, peptides, and proteins such as bovine serum albumin. Where TK work has examined the influence of capping system as a primary or secondary objective, citrate and PVP systems have featured most often.

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[23] See Zhang H et al. (2010) Particle size and pH effects on nanoparticle dissolution. J. Phys. Chem. C. 114: 14876–14884.

[24] For example see: Mollerman B and Hiemstra T (2017). Time, pH, and size dependency of silver nanoparticle dissolution: the road to equilibrium. Environ. Sci.: Nano, 4: 1314-1327.

[25] Refer for example to: Longmire M et al. (2008) Clearance properties of nano-sized particles and molecules as imaging agents: Considerations and caveats. Nanomedicine 3: 703–717.

[26] In respect of NP, the terms 'coating', 'capping' and 'stabilisation system' are typically used synonymously.

Although only two types of AgNP were assessed, the outcomes from a well-conducted oral route study by van der Zande and co-workers (2012) are informative. For AgNP of differing stabilisation system (but equivalent size), Ag concentrations in organs were reported as being highly correlated to the amount of ionic Ag in the NP test article but not to the type of capping system. The authors concluded that GI tract uptake of Ag^+ rather than AgNP was the principle driver of Ag tissue levels. Neither did capping system appear to be a driving parameter on the basis of outcomes from PBTK modelling conducted by Bachler et al., 2013. Some other authors have ascribed clear effects to capping system via inter-study comparisons of TK parameters (particularly in relation to tissue distribution patterns). However, this approach is questionable given the experimental variables between the various studies. Systematic evaluation of different AgNP capping systems within a single investigation is a more secure approach. One such study has been published by Pang et al. (2016) based on single dose i.v. administration of either citrate, PVP, PEG or BPEI capped AgNP of approximately comparable size. Although the authors concluded that major differences were apparent between the various AgNP types, the assessment of this reviewer is that the TK parameter differences which could be clearly attributed to capping system were relatively few, and there was more commonality than made clear in the publication. No similar comprehensive study was identified for the oral route.

Overall, the current weight of evidence is that the AgNP capping system does not seem to have a major influence on TK for a given size of AgNP. Where differences do exist, these are most likely to be associated with capping systems which influence interactions with proteins, or else for certain atypical charge states (as is the case with BPEI capping).

Influence of co-existent ionic silver fraction on AgNP TK

It is clear that a substantial number of published reports concerning the TK of AgNP have not evaluated how much ionic Ag fraction contaminates the AgNP test articles, including that which is: (i) present in the original supplied or synthesised AgNP; or (ii) that formed during storage of stock solutions, or else in dosing solutions which are not freshly prepared on the day of dosing. Work published elsewhere[27] has clearly demonstrated the potentially significant magnitude of ionic Ag formation which may occur, for example on protracted storage (with release values up to 90% being reported) and the variability in Ag^+ release dependent on certain intrinsic AgNP properties and also the conditions of incubation (duration of storage, medium pH etc.). As an illustration of the degree of Ag^+ formation which may occur and its associated kinetics, see the following Figure:

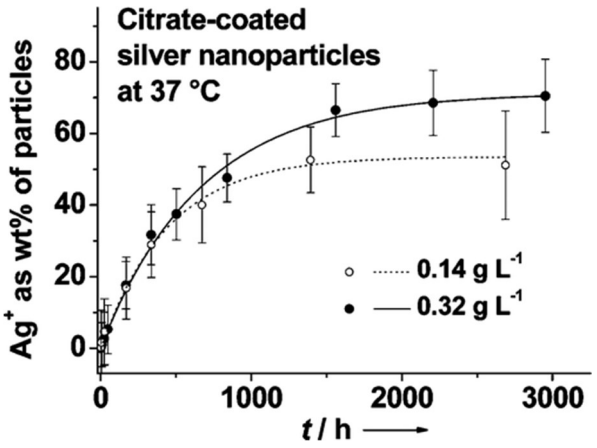


Figure 1. Ionic Ag release from AgNP. 85 nm AgNP; excerpted from Kittler et al., 2010.

[27] Kittler S et al. (2010) Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chem. Mater. 22: 4548-4554.

Where ionic Ag content has been measured in TK investigations, the content can be appreciable and be subject to rapid dynamic change (e.g. see Loeschner et al., 2011; van der Zande et al., 2012). It is the opinion of this reviewer that this variable is likely to be an important experimental confounder and a potential explanation—at least in part—for some apparently divergent TK findings on AgNP. In terms of relevance to more massive (bulk) forms of elemental Ag, it would be predicted that Ag dissolution characteristic will in part relate to dimensional considerations, and therefore that more massive forms of elemental Ag are less likely to generate high concentrations of Ag^+ compared to AgNP (see also previous sub-section).

In interpretation of published work, it is recommended that greater weight (i.e. robustness confidence) be given to studies on AgNP where the possibility of contaminating Ag^+ influence has been properly evaluated. In relation to future studies, proper measurement and control for the presence of Ag^+ fraction should be a fundamental design feature of any new TK study on elemental Ag (irrespective of whether the test article is a nanoscale or more massive form). For instance this would include reliance on freshly prepared dosing solutions and also time-course analytical monitoring of ionic Ag levels in any stock solutions.

Feasibility of applying bioelution modelling to elucidate Ag oral bioavailability

In the case of Ag, some expert reviewers have concluded that several challenges exist in the application of the type of bioelution approaches commonly used to estimate the bioavailability of other non-ferrous metals. For instance, refer to the commentary in the REACH registration dossiers for silver substances. These begin with physical parameter influences where it has been shown that the dissolution behaviour of metallic silver (particularly nanoforms) can be influenced by material characteristics such as particle size distribution, aggregation or agglomeration state, and the presence of surface coatings[28]. In terms of the relevance of solution chemistry, emphasis has been placed on the presence of chloride ions in various G.I. tract compartments (particularly gastric), the consequent formation of poorly soluble silver chloride or silver chloride complexes and related inferences on the limiting impacts on the concentration of free silver ions which are then available for absorption. However, with the emergence of further publications on this matter, it is now clear that the behaviour of Ag species in various physiological milieu is much more complex and multi-factorial, both in the case of Ag^+ and AgNP. A variety of chemical and biochemical processes can influence the absorption characteristics of Ag forms as detailed in references such as Liu and Hurt, 2010[29]; Liu et al., 2012; Walczak et al., 2012; Levard et al., 2012[30]; Zhang, 2013; Bachler et al., 2013; Juling et al. 2016; and Kaiser et al., 2017[31]. These phenomena are summarised (in part and with some simplifications) in Figure 2.

[28] See for example: Zhang H (2013) Application of silver nanoparticles in drinking water purification. Open Access Dissertations. Paper 29. http://digitalcommons.uri.edu/oa_diss/29

[29] Liu J and Hurt, RH (2010). Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ. Sci. Technol. 44: 2169–2175.

[30] Levard C et al. (2012) Environmental transformations of silver nanoparticles: impact on stability and toxicity. Environ Sci Technol. 46: 6900–6914.

[31] Kaiser JP et al. (2017) Cytotoxic effects of nanosilver are highly dependent on the chloride concentration and the presence of organic compounds in the cell culture media. J Nanobiotechnol. 15: 5.

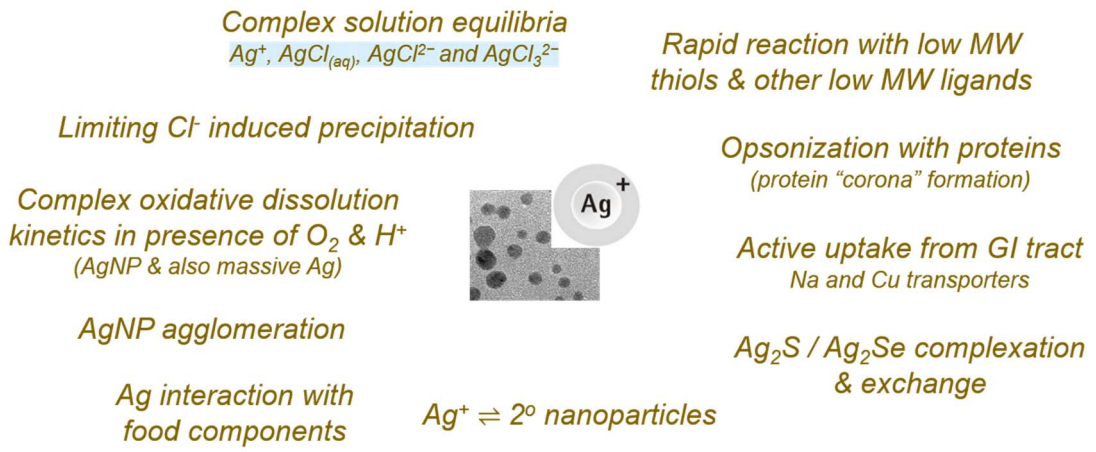


Figure 2. Transformations and interactions of Ag species which have been identified in physiological media and can impact on Ag bioavailability.

Due to the complexities and inter-relationships of these processes, it is concluded that there is very limited prospect that simple bioelution systems can be used to reliably model Ag bioavailability (in terms of mammalian exposure contexts). Instead it is recommended that emphasis be given to the conduct of further well-designed in vivo TK studies, possibly augmented by information from PBTK models. Refer also to further commentary on the latter approach which is provided in the following section "Physiologically-based TK models specifically relevant to Ag".

Physiologically-based TK models adapted to Ag

A physiologically based toxicokinetic model (PBTK) has been developed specifically for Ag. The originators are a team at ETH Zurich, Institute for Chemical and Bioengineering (Zurich, Switzerland). For a related key publication refer to Bachler et al. (2013); as well as Juling et al. (2016). An initial assessment suggests that this approach may be a promising adjunct to the use of in vivo TK studies in order to derive TK parameters for various Ag forms (particularly for gap filling within a grouping of Ag substances).

It has already been questioned as to whether conventional bioelution models have utility in the case of Ag, due to the complexity of its species equilibrium behaviour within the surrogate physiological media, and over doubts that multiple complex interactions e.g. with biological thiols and proteins can be accounted for in such simple systems (refer to the previous sub-section). Data mining of available TK and Ag speciation studies (e.g. Bachler et al., 2013; Walczak et al., 2013; Juling et al., 2016; and Liu et al., 2012) suggests another reason to not place much reliance on bioelution studies. It is difficult to incorporate corrections in bioelution models to account for the formation of secondary NP from ionic Ag and the consequent influence of these species on GI tract uptake (and subsequent processes of distribution and elimination etc.). Use instead of in vivo TK work directly circumvents such issues. However, sophisticated PBTK models, specifically calibrated for Ag parameters, represent another possible approach given that corrections for secondary NP formation can be integrated.

Availability of TK information on silver chloride (AgCl)

This compound has been the test article selected for some studies on the mechanisms of Ag-induced reproductive effects. In particular, those linked to the axis of copper homeostasis.

No reliable TK data using AgCl as the test substance were identified.

Studies relevant to transplacental and embryo-fetal TK

Appendix 2 contains a tabular listing of selected studies which variously provide information on: (i) maternal TK, including detected Ag levels in the placenta; (ii) transplacental Ag kinetic data; and (iii) embryo-fetal TK.

The in vivo investigations were performed in rodents (either rats or mice). Most were based on oral or parenteral (i.v. or i.p.) administration of the Ag test articles. One inhalation route study and also an intratracheal instillation study have been included for completeness.

Salient experimental details for each report are provided as part of the listing (note that in the case of AgNP, the test articles varied between capped and uncapped types). Based on the published datasets, transplacental Ag translocation values have been calculated where appropriate.

INDIVIDUAL STUDY ASSESSMENTS

The following section contains evaluations of individual studies relevant to TK which include:

- Descriptions of salient experimental procedures.
- Summaries of findings reported in the publication.
- Interpretation of TK parameters, including TK parameter derivations where these have been separately derived from data presented in the reports.
- Commentary on the relevance of the study findings, e.g. to the TK profile of Ag substances, or to the interpretation or design of mammalian toxicity studies.
- Study robustness and scientific reliability evaluations.

Refer to Table 1a-c for the bibliographic citation details for each of the publications. The individual study assessments are presented in the same order as in these Tables.

It is important to recognise that the in vivo TK studies cover a range of experimental designs; e.g. oral and parenteral administration routes, single versus repeated dose, and divergences in the precise approaches to the measurement of Ag in tissues. Some of these differences are highlighted in the summarised study descriptions and in related commentary, but it is recommended that the reader should also consult the original publication for more detail.

Barraclough and Cotton, 2017 (4-week study)

The study was a combined toxicity and TK assessment and is an ESTF proprietary report.

Test article: Silver nitrate ($AgNO_3$)

Species: Rat **Strain: Wistar Han**

Both sexes (non-pregnant females).

Age: 7 to 8 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): Males ~190 – 250 g; Females ~130 – 175 g.

Oral (gavage) **Repeated dosing: daily** **Duration: 28 days**

$AgNO_3$: 20; 50; 100 mg/kg bw/d **~13; 32; 64 mg/kg bw/d as Ag equiv.**

Ag levels in <u>plasma</u>

TK determinations: d1 and d27 (Week 4). Composite group sampling design, i.e. semi-serial use of animals in sub-groups (n=3) for designated timepoints.

Timepoints: Pre-dose; 1, 2, 4, 6, and 24 hours post-dose.

Ag digestion/analysis methodology:

Performed under GLP Non-digestion LC-tandem MS

<u>(A) TK data as reported in the publication</u>

Refer to the following excerpted summary Tables from the report covering TK investigations conducted on d1 and at termination (d27):

Day 1	Group 2 (20 mg/kg)		Group 3 (50 mg/kg)		Group 4 (100 mg/kg)	
	Male	Female	Male	Female	Male	Female
C_{max} (ng/mL)	261	432	383	503	404	486
t_{max} (h)	4	4	2	4	6	4
$t_{½}$ (h)	NR	NR	29.5	NR	NR	NR
$AUC_{(0-t)}$ (ng.h/mL)	4530	6220	7060	7980	8910	10900
$AUC_{(0-\infty)}$ (ng.h/mL)	NR	NR	16800	NR	NR	NR
C_{max}/D	13.1	21.6	7.67	10.1	4.04	4.86
$AUC_{(0-t)}$/D	227	311	141	160	89.1	109

NR = No Result Calculable

Week 4	Group 2 (20 mg/kg/day)		Group 3 (50 mg/kg/day)		Group 4 (100 mg/kg/day)	
	Male	Female	Male	Female	Male	Female
C_{max} (ng/mL)	572	925	642	823	1190	1300
t_{max} (h)	6	6	1	6	1	1
$t_{½}$ (h)	NR	NR	29.7	NR	NR	NR
$AUC_{(0-t)}$ (ng.h/mL)	9400	17200	12400	15900	17700	19500
$AUC_{(0-\infty)}$ (ng.h/mL)	NR	NR	28900	NR	NR	NR
C_{max}/D	28.6	46.3	12.8	16.5	11.9	13.0
$AUC_{(0-t)}$/D	470	861	249	318	177	195
RA C_{max}	2.19	2.14	1.67	1.64	2.94	2.68
RA $AUC_{(0-t)}$	2.07	2.77	1.76	1.99	1.99	1.78

NR = No Result Calculable

In relation to toxicity outcomes from the 4-week repeat dose study, the key TK parameters derived from the d27 (Wk4) measurements for the defined No Observed Adverse Effect Level (NOAEL) of 100 mg/kg/day [64 mg/kg bw/d as Ag equiv.] were reported to be as follows:

C_{max}	1190 / 1300 ng/mL (m/f)
$AUC_{(0-t)}$	17700 / 19500 ng.h/mL (m/f)
Elimination half-life ($t_{1/2}$)	N/A (only calculable for males of mid-dose group)

(B) TK data assessment and analysis

Basis: Raw data; from assessment of the TK investigation which was associated with the main repeat dose toxicity study final report (pp. 587-621). Confidence Level (systemic exposure parameters) = HIGH (raw data available).

1. The opportunity was taken to verify some key TK parameters, including those originally calculated by the investigators using the pharmacokinetic analysis package Phoenix WinNonlin®. In particular, $AUC_{(0-t)}$ values were re-calculated using a log-linear trapezoidal analysis. For the d1 TK dataset, the analysis showed good correspondence with the originally reported values (see Table below).

d1 Dataset	Dose level (Ag equiv. values)			
	LD 13 mg/kg bw/d	MD 32 mg/kg bw/d	HD 64 mg/kg bw/d	Units
C_{max} – males	PR	PR	PR	ng/mL
C_{max} – females	PR	PR	PR	ng/mL
t_{max}	PR	PR	PR	h
t_{max}	PR	PR	PR	h
$AUC_{(0-t)}$ – males	4571 (*4530*)	7085 (*7060*)	8937 (*8910*)	ng.h/mL
$AUC_{(0-t)}$ – females	6236 (*6220*)	8002 (*7980*)	10914 (*10900*)	ng.h/mL
Remarks		≡ to Sprando et al. high-dose group		

PR = Determined to be as per the data value in the report. () = Italicised figures are values cited in the report.

2. As per the report, group mean blood Ag C_{max} were confirmed to occur between 1-6 h (for d1) and 2-6 h (at d27).
3. Based on derived $AUC_{(0-t)}$ there is evidence of non-linear systemic exposure[32]. A partial plateauing effect occurs, particularly in respect of the female dose groups (mid-dose and high-dose). The non-linear characteristic (i.e. relative systemic exposure diminishing versus absolute dose administered) was confirmed by dose-normalised group comparison (calculation of $AUC_{(0-t)}$ / D). After both single and multiple administrations, increases in mean exposures were generally sub-proportional with respect to dose. This is indicative of either saturation of absorption (most likely); or an increase in clearance at higher doses (unlikely); or an increased volume of distribution at the higher dose levels (unlikely).

[32] If non-linear AUC values between doses are apparent, this is evidence of saturation of one or more key kinetic processes, i.e. reversion to zero-order kinetics.

4. Contrary to the report conclusions, there did appear to be a gender-related difference in the achieved absolute $AUC_{(0-t)}$ values when intra-group comparisons were made, whereby systemic exposure was consistently greater in female sub-groups at both d1 and d27:

$AUC_{(0-t)}$ ratio females : males (%)			
	Lose-dose	Mid-dose	High-dose
d1	137	113	122
d27 (Wk 4)	183	128	110

   This effect was non-linear in respect of increasing dose (for reasons described in Point '3'). Other investigators have described similarly greater exposure of female animals relative to males that received the same dose-level of Ag (e.g. Boudreau et al., 2012; 2016).

5. Based on the two TK datasets, systemic Ag accumulation was evident at the d27 timepoint based on comparison of group mean values evident at d1, viz. ratios of 1.64 – 2.94 (for C_{max} and 1.76 – 2.77 AUC(0-t), respectively. No gender-linked or dose-dependency was evident in respect of this finding.

(C) Relevance

1. The study is relevant to determination of basic TK parameters connected to systemic exposure via the oral route for a soluble Ag form ($AgNO_3$). **Study rank for this purpose: KEY**.

2. The TK results are considered to be relevant to interpretation of Sprando et al./Babu et al. studies[33] and to the EPMF EOGRTS design for the following reasons:
   a. Although the test article differed in this study ($AgNO_3$) versus that selected by Sprando et al. (AgAc), both are ionic Ag compounds with similar rank water-solubility. As described in the 'Conclusions' section, it should be noted that there are grounds to conclude that $AgNO_3$ possesses somewhat lower bioavailability when orally administered to rats than AgAc.
   b. The TK study was performed in the same species (rat; young adult; both sexes).
   c. The route and dosing regimen used is relevant (oral route; repeated dosing).
   d. The dose levels intersect those of interest. In terms of normalised Ag equivalent administered dose, the mid-dose (32 mg Ag/kg bw/d) in this study has approximate correspondence to the high-dose in the Sprando et al./Babu et al. investigations (26 mg Ag/kg bw/d).
   e. Though the duration of dosing and the linked terminal TK assessment was only 4 weeks whereas OGRTS/EOGRTS designs typically include adult animal dosing for up to 10 weeks, there are mitigating considerations. It would be expected that steady-state kinetics in relation to systemic exposure would be evident by 4 weeks.

[33] Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation: Not applicable.
2. The TK segment was performed according to GLP.
3. Test article stability and homogeneity checks were performed.
4. Concentration verification for dosing solutions was conducted.
5. Quantitation of silver was performed by LC-MS/MS rather than the more usually applied methodology of ICP-MS. However, the limit of quantitation achievable via this alternate approach is acceptable. The Ag digestion and analytical methods were supported by appropriate calibration, quality control and reproducibility studies.
6. The group sizes were considered to be adequate for performance of a TK study.
7. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent (even for high-dose animals).

Boudreau et al., 2012 (Single dose TK study)

Full citation details: Boudreau MD (2012) An evaluation of the toxicological effects of discrete sizes of silver nanoscale particles (AgNP) in the Sprague Dawley rat. Presentation at the BfR conference "Nanosilver"; 9 February 2012.

Test article: Silver acetate (AgAc) **Silver NP (10 nm / 75 nm / 110 nm); citrate-capped**

Species: Rat **Strain: Sprague Dawley**

Both sexes (non-pregnant females).

Age: 7 weeks old at the start of dosing.

Bodyweight (at commencement of dosing: males 325 g; females 200 g.

Oral (gavage) / Intravenous (bolus; via tail vein) **Single dose**

Oral: AgAc – 10 mg/kg bw AgAc / 6.5 mg/kg bw as Ag equiv. AgNP – 10 mg/kg bw.

IV: AgAc – 3 mg/kg bw AgAc / 1.95 mg/kg bw as Ag equiv. AgNP – 3 mg/kg bw.

Ag levels in <u>whole blood</u> (EDTA anticoagulant)

TK determinations: d1

Timepoints: 0, 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h

Ag digestion/analysis methodology:

ICP- MS

<u>(A) TK data as reported in the publication</u>

1. Blood Ag concentration data are detailed in '(B) TK data assessment and analysis'.
2. Oral route: elimination half-time ($t_{1/2}$) was estimated as circa 24 h (for males and females).
3. Oral route: group mean T_{max} values were interpolated as 12 h (m/f).

<u>(B) TK data assessment and analysis</u>

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = MEDIUM (raw data unavailable; interpolations made from the publication figures).

1. For the 3 sizes of AgNP studied (10, 75, 110 nm) increased $AUC_{(0-t)}$ correlated monotonically with decreasing AgNP size; therefore the 10 nm AgNP was apparently the most bioavailable AgNP size via the oral route. However, the precise influence of particle number-dose cannot be ascertained for each of the AgNP test articles based on the available data in the publication.

2. Oral route $AUC_{(0-t)}$ – and hence achieved systemic exposure via that route – was greater for the soluble Ag form (AgAc) compared to all sizes of AgNP. To examine the magnitude of this difference based on a nominal one-day timespan, oral $AUC_{(0-t)}$ values up to 24 h were compared for AgAc and AgNP-10 nm, the latter being the AgNP with the greatest apparent bioavailability. Relative AUC ratios were then corrected for administered Ag equivalent dose. ***Conclusion:*** systemic exposures over this period were approximately 2.7x (males) to 3-fold (females) higher in the case of AgAc.

	$AUC_{(0-t)}$ ng.h/mL		**Ratio AUC values AgAc : AgNP[10 nm]**		**AUC ratios corrected for Ag equivalent dose AgAc : AgNP[10 nm]**	
	m	f	m	f	m	f
AgAc	2770	6060	1.74	1.92	2.67	2.96
AgNP-10nm	1595	3155				

3. Mean C_{max} and $AUC_{(0-t)}$ values were greater in the case of females, both for an ionic form (AgAc) and in the case of AgNP. ***Conclusion:*** for AgAc, this was congruent with findings in the study by Barraclough and Cotton (2017) related to another ionic form ($AgNO_3$).

4. After a single oral dose of AgNP (10 mg Ag/kg bw/d) or AgAc (6.5 mg Ag/kg bw/d) T_{max} values were similar, i.e. 12 h. ***Conclusion:*** for the ionic form, AgAc, the observed T_{max} value after a single oral dose was significantly longer than that determined in the Barraclough and Cotton (2017) report on $AgNO_3$ when related to the most comparable dose group in that study, viz. 13 mg Ag/kg bw/d.

5. Bioavailability by the oral route was calculated from the respective oral and i.v. $AUC_{(0-t)}$ datasets for AgAc, AgNP-10 nm (the AgNP type with the greatest apparent systemic exposure) and AgNP-110 nm (the type with the least apparent systemic exposure). Values were derived based on administered equivalent Ag doses. It is inferred that the oral bioavailability of all these Ag forms is low and only limited absorption occurs following a single oral dose administration, i.e. in the order of 4%, or less. However, based on this dataset, approximately similar bioavailability was evident when comparing AgNP-10 nm to AgAc at closely matched doses (refer to Table); actually being somewhat greater for AgNP-10 nm. The corresponding F values for AgNP-110 nm (see Table) and AgNP-75 nm (not shown) were lower and diminished in a manner which correlated with increasing NP size.

	$AUC_{(0-t)}$ ng.h/mL (males)		**$AUC_{(0-t)}$ ng.h/mL (females)**		**Bioavailability [F] %**	**Bioavailability [F] %**
	p.o.	i.v.	p.o.	i.v.	males	females
AgAc 6.5 mg Ag/kg bw p.o. 1.95 mg Ag/kg bw i.v.	4830	64710	10990	127615	2.2	2.6
AgNP-10nm 10 mg Ag/kg bw p.o. 3 mg Ag/kg bw i.v.	3640	31195	6210	57815	3.5	3.2
AgNP-110nm 10 mg Ag/kg bw p.o. 3 mg Ag/kg bw i.v.	815	31060	1740	30970	0.8	1.7

6. In respect of the i.v. route datasets (for AgAc and AgNP-10 nm), there was a clear disparity in group mean blood concentrations between measurements for female and male animals for the 0 h timepoint, with lower values being evident in respect of males. The reason for this anomaly is unclear, e.g. whether it is an experimentally introduced artefact. There is some corresponding skewing of the AUC estimates.

7. The i.v. route blood concentration kinetics for AgAc show a biphasic character with an early rapid elimination phase followed by a protracted plateau phase after 40 h. As silver is known to form inert complexes with blood compartment proteins[34], such as albumin and macroglobulins, it is hypothesised that this may be retarding its full clearance.

(C) Relevance

1. The study is pertinent to determination of basic TK parameters connected to systemic exposure via the oral route (and i.v. route) for elemental Ag (several AgNP forms) and a soluble Ag form (AgAc). **Study rank for this purpose: KEY**.

2. The investigation has some relevance to interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. The TK study on AgAc was performed in the same species (rat; young adult; both sexes). Route and dosing regimen are relevant (including the oral route); with dose levels which intersect those of interest[35]. However, the TK investigation is based on administration of a single dose rather than repeated dosing, which needs to be borne in mind when making direct extrapolations, i.e. steady-state TK conditions will not have been achieved.

3. This study is notable for its demonstration of a comparatively high oral bioavailability estimate a type of AgNP, viz. AgNP-10 nm (citrate capped). The derived values at 3.5% for males and 3.2% for females (refer to previous commentary) are amongst the highest established to date for any AgNP form. The larger AgNP which were administered (viz. AgNP-75 nm and AgNP-110 nm) had lower, but not negligible oral bioavailability.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures were evaluated as robust. This included quantitation of silver in the AgNP stock suspensions that was in the ionic form.

2. Test article stability and homogeneity checks were performed. Concentration verification for dosing solutions was conducted.

3. The nature of the i.v. dosing formulation is not stated in the abbreviated study report. In respect of avoidance of Ag precipitation issues with i.v. dosing preparations, e.g. caused due to use of isotonic saline, the FDA group's standard practice is known to be the use of a 5 % isotonic glucose solution. It is assumed that this study adhered to this practice.

4. The group sizes were considered to be adequate for performance of a TK study.

5. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

6. The study was non-GLP.

[34] Liu J et al. (2012).

[35] Sprando RL et al. (2017)/Babu US et al. (2016): Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

van der Zande et al., 2012 (4-week study)

Test article: Silver nitrate ($AgNO_3$) **Silver NP (15 nm); PVP-capped**

Silver NP (18 nm); polyoxyethylene glycerol trioleate (4%) and Polysorbate 20 stabilised (4%) [OECD reference nanoform]

Species: Rat **Strain: Sprague Dawley**

<u>Single sex</u> only (males).

Age: ~7 weeks old at the start of dosing (assuming standard acclimatisation).

Bodyweight (at commencement of dosing): Males ~245 g.

Oral (gavage) **Repeated dosing: daily** **Duration: 28 days (4-wk)**

$AgNO_3$: 9 mg/kg bw/d **~6 mg/kg bw/d as Ag equiv.**

AgNP 15 nm (PVP-capped) & 18 nm (surfactant stabilised): 90 mg/kg bw/d.

Ag levels in <u>whole blood</u> / faecal Ag levels / terminal Ag tissue levels

TK determinations: Ag in blood (weekly intervals; d0, d7, d14, d21) at 5h post-dose. Faecal Ag levels (weekly intervals; pre-dose collection). Terminal Ag in blood sample (d29) and Ag in various tissues. Wash out groups: Ag in blood and faecal Ag levels (as above) to either d36 or d84, at which timepoint terminal tissue samples were obtained for Ag determination.

Ag digestion/analysis methodology:

Nitric acid[36]-microwave digestion / AAS

<u>(A) TK data as reported in the publication</u>

1. Blood Ag levels in the $AgNO_3$ group were consistently significantly higher than that of the AgNP groups throughout the treatment period (i.e. circa 7- to 10-fold greater). The investigators concluded that there was a result of a much higher uptake of Ag following administration of the soluble Ag form ($AgNO_3$) compared to either type of AgNP. Refer to excerpted Figure 4A.
2. For the two forms of AgNP, Ag concentrations in organs were reported as being highly correlated to the amount of ionic Ag in the dosed NP test article. The authors concluded that GI tract uptake of Ag^+ rather than AgNP was the principle driver of Ag tissue levels.
3. Recovery group ("wash-out" group) studies demonstrated that blood Ag levels rapidly declined for all treated groups upon cessation of treatment (see publication Figure 4A), being reduced by more than 50% by d36 (1 week after the final dose) with full clearance being evident by d86. In the case of both $AgNO_3$ and AgNP treated animals, Ag clearance from most organs was evident by 8 weeks post-treatment (though Ag was still detectable in the brain and testis).

[36] Digestion of stock/dosing solutions prior to analysis was instead performed with a chloride-rich acidic medium to facilitate AgNP dissolution.

4. Ag tissue distribution data are addressed in the next section. A notable observation was that levels of Ag associated with the brain and testis were quite marked in the case of both the $AgNO_3$ and AgNP treated rats.

(B) TK data assessment and analysis

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.
2. However, from the blood Ag concentrations obtained at weekly intervals (5h post-dose sample point), it is possible to conclude that Ag levels plateaued for all treated groups, and that there was no evidence of accumulation kinetics. Refer to excerpted Figure (which includes corrections for normalised silver dose for the treated groups):

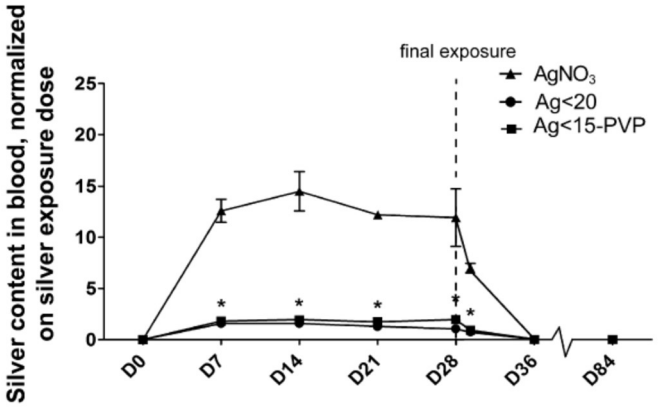


Figure 4A. Silver content in blood (mean (SEM, n = 5) during 84 days. The blood silver content normalized on the daily silver exposure dose is presented as a ratio between the measured silver concentration in blood (µg silver/kg blood) and the daily silver (Ag ions and AgNPs) exposure dose (mg silver/kg bodyweight).

3. In the case of the GI tract, liver, kidney and spleen, the distribution pattern of Ag following either $AgNO_3$ exposure or the two forms of AgNP resembled that reported in other studies on rats orally exposed to ionic Ag forms (such as AgAc or $AgNO_3$) and to AgNP. Refer also to excerpted Figure 6 for the results. At 2-4x blood concentrations, the relative distribution to the testis was greater in this study than has been reported in some other studies, e.g. compared to Lankveld et al., 2010 (based on repeated dosing via the i.v. route). Whereas, appreciable distribution to the testis has been described in a 4-week oral study with AgNP (Kim et al., 2008). Data are also contradictory as to whether Ag distributes into the brain to a significant extent (refer to the Conclusions section of this review and also to commentary in the EPMF EOGRTS Testing Proposal[37]). For instance, low levels of Ag associated with the brain were reported by Kim et al., 2008 and Loeschner et al., 2011 which are not congruent with this study. The underlying reason(s) for the inconsistency is unclear, particularly in the case where investigations with soluble Ag forms are compared. The van der Zande et al. publication lists some of the conflicting studies. In the case of the brain and the testis, it would be relevant to understand whether Ag-associated depots are within the organ or else associated with

[37] Testing proposal: EOGRTS (OECD TG 443) – Silver/silver ionic substances; EPMF, 2018.

the endothelial compartment (as discussed further in the Conclusions section, this represents a current research need).

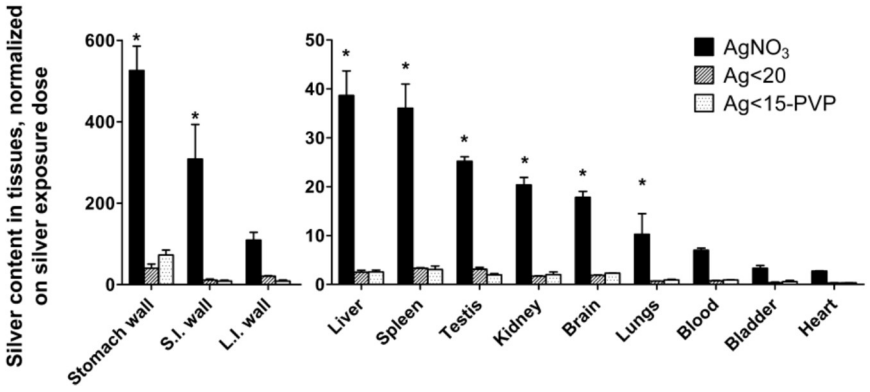


Figure 6. Silver content in tissues (mean (SEM, n = 5) at day 29. (A) The tissue silver content normalized on the daily silver exposure dose is presented as a ratio between the measured silver concentration in tissue (µg silver/kg tissue) and the daily silver (Ag ions and AgNPs) exposure dose (mg silver/kg bodyweight).

4. A speculative possibility is that the greater persistence of detectable Ag in the brain and the testis relative to other tissues could be due to the influence of the blood-brain and blood-testis barriers (and their endothelial compartments) retarding the clearance of intra-organ Ag depots from these specific tissues.

5. The study is notable in its emphasis on investigating the Ag dissolution characteristic (formation of Ag^+) from AgNP stock/dosing form suspensions. For instance, it was determined that whereas the Ag^+ content of the AgNP-18 nm (uncoated) remained low and stable during normal storage period, Ag ion dissolution from the AgNP-15 nm (PVP) was unstable and quickly increased to up to 45% after 7 days. It is inferred that the AgNP-15 nm (PVP) treatment exposed the animals to variable and probably substantial amounts of ionic Ag alongside nanoparticulate Ag. The $AgNO_3$ dose level was selected to equate to the ionic Ag content present in the AgNP-18 nm (uncoated) test article.

6. Single-particle ICP-MS studies performed adjunctively to the tissue distribution TK work demonstrated that tissue-resident AgNP were actually evident in rats treated with the soluble Ag form ($AgNO_3$). This suggests that in situ formation of AgNP from ionic Ag occurs. The formation of secondary NP from ionic Ag forms has been separately confirmed in other work (Juling et al., 2016).

(C) Relevance

1. The study has some relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for elemental Ag and a soluble Ag form (AgAc), but data from a single timepoint blood sampling regimen do not permit conventional AUC estimates.

2. In terms of absorption phase, this study is considered important in confirming steady state kinetics (given it was a 4-week duration dosing period), and due to its information on the interrelationship between AgNP characteristics and TK. **Study rank for this purpose: KEY.**

3. The findings relating to elimination and to Ag distribution pattern are mainly congruent with several other TK studies examining elemental and ionic Ag forms, with the exception of the Ag tissue concentrations in the testis and brain. **Study rank for this purpose: SUPPORTING**. It should be noted that the design included only male animals.

4. The TK results have some relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. An ionic Ag compound was included ($AgNO_3$). The TK study was performed in the same species (rat; young adult) though the design included only male animals. The route and dosing regimen are relevant (oral route; repeated dosing). However, the dosing period duration/terminal TK assessment was only 4 weeks. In terms of normalised Ag equivalent administered dose, the only dose level selected for $AgNO_3$ (~6 mg Ag/kg bw/d) resides between the mid- and high-dose of the Sprando et al./Babu et al. investigations[38]. The findings on Ag retention in the brain and testis do have potential bearing on the EPMF EOGRTS design.

5. The AgNP coating characteristic (PVP-capped versus surfactant-capped) had no major impact on TK parameters. Whereas an important finding from this work was the influence of the associated Ag^+ fraction on AgNP biokinetics.

(D) Study robustness and scientific reliability indicators

1. One of the selected Ag nanoparticles (AgNP-18 nm) is a very well characterised OECD reference material. The capped AgNP (15 nm) was from a commercial source.

2. AgNP characterisation procedures were evaluated as robust (also taking into account the use of an OECD reference material). This included Ag^+ quantitation in the AgNP stock suspensions (between 7-8% for both NP types as freshly-prepared materials, but rapidly escalating on storage of the PVP-stabilised AgNP).

3. Test article stability and homogeneity checks were performed. Concentration verification for dosing solutions was conducted. Assessments for storage-induced artefacts (such as serial measurements of ionic Ag release characteristic) were via appropriate methodologies.

4. The group sizes were considered to be adequate for performance of a TK study.

5. AAS as a detection technique for Ag may lead to relatively poor limit of detection, but the methodology in this study achieved acceptable sensitivity[39]. Nevertheless, it should be noted that the blood samples collected up to d28 were reported as being 'pooled per group' to ensure sufficient material for measurements.

6. Faecal samples were not described as obtained from use of metabolic cages (animals were gang-housed during the treatment period).

7. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

8. The study was non-GLP.

[38] Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

[39] Limit of detection for the AAS analytical technique was stated as 5 µg/kg for blood and 100 µg/kg for tissue samples, GI tract contents, and faeces.

Park et al., 2011 (Single-dose study)

Test article: Silver NP (8 nm); citrate-capped (OECD reference nanoform)

Species: Rat Strain: Sprague Dawley

<u>Single sex</u> only (males).

Age: 7 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): unstated.

Oral (gavage) / Intravenous (bolus; via tail vein) Single dose

Oral: AgNP – 1; 10 mg/kg bw.

IV: AgNP – 1; 10 mg/kg bw.

Ag levels in <u>whole blood</u> (EDTA anticoagulant)

TK determinations: via terminal sacrifice of sub-groups, i.e. not serial individual samples. Sample point was the abdominal aorta.

Sample points: 0, 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h

Ag levels in faeces / urine

Faecal and urinary Ag levels via 24 h collection post-dose.

Terminal Ag tissue levels

Terminal tissues samples were obtained for Ag quantitation at 24 h and 96 h post-dose (comprising a limited tissue set).

Ag digestion/analysis methodology:

Nitric acid/peroxide-microwave digestion / ICP-MS.

<u>(A) TK data as reported in the publication</u>

1. Blood concentration of Ag following oral and i.v. administration are reported as time-course data in Figure 1 and 2, respectively, of the publication. Data relating to systemic availability parameters are summarised as follows:

	i.v. route		Oral route	
	1 mg/kg bw	10 mg/kg bw	1 mg/kg bw	10 mg/kg bw
C_{max} (ng/mL)	1351	5223	83	673
t_{max} (h)	0.17*	48	24	8
$t_{½}$ (h)	NC	99	NC	30
$AUC_{(0-t)}$ (ng.h/mL)	96530	459500	1160	19430
$AUC_{(0-\infty)}$ (ng.h/mL)	199070	NC	NC	21570
C_{max}/D	1351	522	83	67
$AUC_{(0-t)}$/D	96530	45950	1160	1943
Bioavailability (F)			**1.2%**	**4.2%**

NC = Not calculable. *At first sample point 10 min. Units adjusted to hours.

Original TK data were derived using BACalc, 2007, ver. 1.0.0; Korean FDA. Appropriate rounding has been applied to TK value conversions.

2. Terminal Ag in tissue levels are reported in Table II of the publication.

3. Cumulative urinary and faecal Ag content values (to 24 h) are provided in Table III of the publication.

(B) TK data assessment and analysis

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = MEDIUM (raw data unavailable; interpolations made from the publication figures). In any inter-study comparisons, it should be borne in mind that all values are derived from these investigations relate only to male animals.

1. At 4.2%, the calculated bioavailability (F) via the oral route for this form of AgNP (8 nm; citrate-capped), based on a dose of 10 mg/kg bw, was close to that evident in work by Boudreau et al., 2012 on AgNP of comparable dimensions and stabilisation (viz. AgNP-10nm; citrate-capped). In the latter study an oral bioavailability of 3.5% was derived for male animals following an equivalent regimen of a single oral gavage dose of 10 mg/kg bw.

2. In respect of correspondence between the i.v. TK segment here and Boudreau et al., 2012: after appropriate dose normalisation, C_{max} and T_{max} values were reasonably similar for similar size AgNP forms, whilst achieved $AUC_{(0-t)}$ was higher in this study due to a more sustained blood Ag concentration, particularly at 10 mg/kg bw. Via the oral route (at a comparable dose of 10 mg/kg bw), the achieved C_{max} in this study was apparently 5-fold higher as was the $AUC_{(0-t)}$ value; whereas T_{max} was shorter at 8h (versus 12h in Boudreau et al., 2012). The calculated elimination half-life via this route in both studies was reasonably comparable, i.e. 30h here and 24h in the case of Boudreau et al.

3. In terms of systemic exposure considerations, it can be argued that the outcomes from this study are reasonably congruent with that of Boudreau et al., 2012. This includes derived oral bioavailability values which are of the same order. Both studies relate to AgNP of similar dimensions and stabilisation system; though it would have been helpful also to compare the ionic Ag content of the AgNP test articles, this is precluded due to lack of information in Park et al., 2011.

4. The dataset for terminal Ag in tissue concentrations covers only 3 tissues (viz. lung, kidney, and liver) which limits the value of the study in respect of Ag distribution aspects. As would be expected, tissue concentrations from animals dosed via the i.v. route were substantially greater than for animals dosed orally. However, the very high Ag levels detected in the liver at 24 and 96 h for the group receiving 10 mg Ag/kg bw (i.v.) seem anomalous since these exceed the total administered Ag dose several-fold (Table II of the publication).

5. The urinary and faecal Ag content data provided are quite simplistic, e.g. it is not possible to calculate the percentage of administered dose eliminated via each mode. As would be expected, the faecal values were greater in rats dosed via the oral route. For both routes of exposure, the finding that Ag content in the urine is minimal compared to faecal levels is congruent with many other previous TK investigations on Ag.

(C) Relevance

1. The study is relevant to determination of basic TK parameters connected to systemic exposure via the oral route for a form of elemental Ag (a single type of AgNP). Notwithstanding the single dose design, it is one of the few studies with concurrent oral and i.v. dosing regimens which also incorporates serial measurement of Ag in blood. Hence it does permit derivation of oral bioavailability estimates. **Study rank for this purpose: KEY**.

2. It also provides limited information on Ag elimination and distribution. However, there are reservations in respect of the reported Ag concentrations in tissues**. This part of the study should be set aside on reliability considerations (EXCLUDED).** Other superior TK studies exist for this phase.

3. The investigation has limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. The TK study was performed in the same species (rat), albeit in males only; via a relevant route and dosing regimen (incorporating the oral route); and it includes total Ag dose levels which intersect those of interest[40]. However, the test article was not a soluble Ag form but AgNP, and the design was based on a single dose rather than repeated dosing (i.e. steady-state TK conditions are not expected to have been achieved).

4. The absence of certain key procedural details – such as those relating to test article chemical characterisation, dose formulation specifics and related quality controls – represents an impediment to a superior KL ranking for this study. Refer also to the section 'Study robustness indicators'. However, based on the experimental details provided, the study design and its performance is considered to be acceptable, and it should be included in the overall TK database on Ag.

(D) Study robustness and scientific reliability indicators

1. The selected AgNP (8 nm; citrate-capped) test article is a well characterised OECD reference material. The AgNP characterisation procedures listed were evaluated as robust for physical parameters (also taking into account the OECD reference material status). No quantitation of Ag present in the ionic form in the AgNP stock suspensions was described, which is sub-optimal in terms of robustness. The use of an OECD reference nanoform partly mitigates this concern, but fuller interpretation of study outcomes is complicated by the absence of concurrent ionic Ag measurements.

2. Concentration verification for dosing solutions was not reported as performed. No detailed descriptions of the procedures / vehicles used for gavage or i.v. dose formulation are provided in the publication.

3. The group sizes were considered to be adequate for performance of a TK study.

4. For Ag concentration determinations, the preparative microwave-based technique involved digestion with hydrogen peroxide/nitric acid mixture rather than nitric acid alone, which is a less commonly used technique. Data from internal standards and recovery values were not provided. However, the reported LOD via ICP-MS analysis showed high sensitivity (0.001 ppb).

[40] Sprando et al./Babu et al.: Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

5. There are issues with the Ag concentration in tissue dataset where some apparent recoveries exceed the total administered dose.

6. Urine and faecal samples were obtained via the use of individual metabolic cages in a manner which minimised the potential for contamination.

7. Overt toxicity of a nature considered sufficient to interfere with TK outcomes did not occur.

8. The study was non-GLP.

Boudreau et al., 2016 (13-week study)

Test article: Silver acetate (AgAc) **Silver NP (10 nm / 75 nm / ~110 nm); citrate-capped**

Species: Rat **Strain: Sprague Dawley**

Both sexes (non-pregnant females).

Age: 7 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): males 325 g; females 200 g.

Oral (gavage) **Repeated dosing: daily** **Duration: 90 days (13-wk)**

AgAc: 100; 200; 400 mg/kg bw/d **65; 130; 260 mg/kg bw/d as Ag equiv.**

AgNP (10, 75, or 110 nm): 9; 18; 36 mg/kg bw/d

Ag levels in <u>whole blood</u> (EDTA anticoagulant)

TK determinations: Study Weeks 1 (d1), 4, 8, and 12.

Timepoints: 0, 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h.

Terminal Ag tissue levels

13 Wk timepoint.

Ag digestion/analysis methodology:

Tissue digestion methodology was not explicitly stated but is inferred to have been based on a nitric acid-microwave accelerated technique / Analysis was by ICP- MS.

<u>(A) TK data as reported in the publication</u>

1. In respect of TK data examining systemic exposure and bioavailability, the publication cross-refers to results obtained in a preliminary TK study by the oral and i.v. routes which utilised lower doses of Ag (see also Boudreau et al., 2012).
2. Week 1 and Week 12 data on tissue distribution of Ag following administration of the various AgNP and AgAc are presented in Supplementary Table 3 (see Appendix 1 of this report) and are summarised in Tables 5-7 of the main publication. Results for various regions of the gastrointestinal tract and associated lymph nodes are presented, along with Ag concentrations in the liver, spleen, kidneys, heart and <u>uterus</u>.
3. The absolute concentration of silver in tissues showed significant AgNP size-dependent relationships, with the rank order being 10 nm >>75 nm >110 nm.

<u>(B) TK data assessment and analysis</u>

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data (but see also the data available in Boudreau et el., 2012).

1. The absence of a data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.

2. Figure 5 of the publication includes the depiction of Ag content in the blood for rats administered either AgNP or AgAc; the TK timepoints being Week 1 and also Week 12 (near-terminal). The authors do not explicitly state whether the Ag in blood values are group mean total collected serial fractions results or else C_{max} values. However, several broad conclusions can be drawn from these datasets:

    a. For the 3 forms of AgNP (10 / 75 / 110 nm), and for all dose levels and both sexes, there is no evidence of Ag accumulation when Week 1 and Week 12 datapoints are compared. In the case of AgAc, comparisons of Week 1 vs Week 12 values are only possible for the low- and mid-dose groups (65 or 130 mg Ag/kg bw/d) – again there is no evidence of Ag accumulation in the blood. A preliminary study by Boudreau et al. (2012) had estimated that the elimination half-life for AgNP and AgAc was approximately 24 h, and these results add support to the supposition that both Ag forms are cleared relatively rapidly from the blood in a manner which is invariant with the duration of dosing.

    b. In keeping with the findings of Boudreau et al. 2012, group mean blood Ag levels of AgNP-10nm were comparatively higher than those of AgNP-75 or 110nm (Figure 5A and B) possibly indicating a higher relative bioavailability for the smallest AgNP. However, the precise influence of relative particle number-dose cannot be ascertained.

    c. Silver concentrations were significantly higher in the blood of rats which received AgAc compared with those of rats exposed to AgNP (Figure 5C and D), even allowing for differences in total administered Ag dose. This difference was also evident in the tissue Ag concentrations (see Appendix 1), whereby Ag concentrations in multiple tissues were correspondingly greater in rats which received AgAc, even after normalisation for equivalent Ag dose (with the effect generally being greater for females than males for solid organs).

3. Ag in tissue concentrations at Wk 12 were not dissimilar to other distributional datasets for ionic Ag and AgNP. In rank order, the uterus was an intermediate site of distribution; the measured Ag levels increased in a dose-dependent manner in the case of AgAc and AgNP-10 nm. However, the relationship for the larger AgNP (75 / 110 nm) was dissimilar with peak uterine Ag concentrations being evident at the mid-dose (18 mg Ag/kg bw/d). The reason for this non-linear relationship with dose is unexplained.

(C) Relevance

1. The study contains only limited data which are pertinent to the determination of basic TK parameters connected to systemic exposure via the oral route for elemental Ag and a soluble Ag form (AgAc).

2. The tissue distribution results (including achieved blood levels) are pertinent to the interpretation of the Sprando et al./Babu et al. studies[41] and to the EPMF EOGRTS design. The ionic Ag compound studied was the same, viz. AgAc. The TK investigation was performed in the same species (rat; young adult; both sexes). Route and dosing regimen were equivalent (oral route; repeated dosing). Duration of dosing (and the terminal TK assessment at 12 weeks) approximately equates to the parental generation

[41] Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

pre-mating dosing period applicable to E/OGRTS. However, the following caveats should be taken into account:

a. Administered doses of AgAc (65; 130; 260 mg/kg bw/d as Ag equiv.) were markedly greater than those in the Sprando et al./Babu et al. studies.

b. For the AgAc dose levels producing evident toxicity and/or biome impacts (i.e. particularly the high-dose and mid-dose), the possibility of disturbance of normal gastrointestinal function and consequent effects on Ag TK cannot be disregarded in this study.

3. The most useful TK datasets contained within this publication are considered to be the Ag distribution values in various tissues (Week 1 / Week 12), particularly in respect of the low-dose group, and in particular the Week 12 data on tissue distribution of Ag. **Study rank for this purpose: KEY**. Information on concentrations of Ag in the uterus are available, but unfortunately not in the testes or brain. This tissue distribution dataset is particularly notable given that the investigation is the only robust TK study in the rat–including an ionic Ag reference substance–with an extended dosing period to 13 weeks. Hence it is useful in respect of E/OGRTS design and interpretation (with the aforementioned caveat that the lowest dose level studied was quite high at 65 mg Ag/kg bw/d). As stated previously, the treatment group results are presented in detail in Supplementary Table 3 (duplicated in Appendix 1 of this report), with summaries in Tables 5-7 of the main publication.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures were evaluated as robust. This included quantitation of silver in the AgNP stock suspensions that was in the ionic form.

2. Test article stability and homogeneity checks were performed. Concentration verification for dosing solutions was conducted. Assessments for storage-induced artefacts (such as serial measurements of ionic Ag release characteristic) were properly conducted.

3. The group sizes were considered to be adequate for performance of a TK study.

4. Overt toxicity of a nature considered sufficient to interfere with TK outcomes did occur in AgAc treated groups. This particularly included toxicities and/or gut microbiome disturbances at the mid- and high-dose levels which may have impacted on oral absorption kinetics in these animals. Some interpretative caution is therefore advisable in respect of TK findings for these treated groups.

5. The study was non-GLP.

Klaassen, 1979

Test article: Silver nitrate (^{110}Ag admixture)[42]

Species: Multiple (refer to table below)

Species	Strain	Sex	Age	Bodyweight	Dose level (i.v. route¤; single dose administered)
Rat	**Sprague Dawley**	**Single sex only (males)**	**NS***	**200 – 300 g**	Biliary-urinary elimination: 0.1; 0.3 mg Ag/kg bw Time-course of biliary elimination: 0.01; 0.03; 0.1; and 0.3 mg Ag/kg bw
Rabbit	**-**	**Single sex only (males)**	**NS***	**1.5 – 2.5 kg**	Biliary elimination characteristic: 0.1 mg Ag/kg bw
Dog (Mongrel)	**-**	**Single sex only (males)**	**NS**	**8 – 14 kg**	Biliary elimination characteristic: 0.1 mg Ag/kg bw

NS = not stated. * inferred to be young adult animals. ¤ administered via femoral vein.

Ag levels in faeces and urine

TK determinations: urine and faeces collection (24 h period) up to 7 days post-dose.

Ag levels in bile and blood

TK determinations: Animals were cannulated via the femoral artery for administration of Ag, and for the collection of bile and also blood samples at intervals up to 2h post-dose. Both whole-blood and plasma were analysed for ^{110}Ag concentrations.

Terminal Ag tissue levels

Terminal tissue samples were obtained from rats at 2h post-dose. The tissues evaluated by scintallography comprised the liver, kidney, pancreas, intestine (location not further specified), stomach, brain, spleen, heart, bone (tibia), testes, muscle (soleus/gastrocnemius), and lung.

Ag digestion/analysis methodology:

Digestion was not required (gamma spectrometry was the detection technique).

Analytical methodology details are omitted from the publication.

(A) TK data as reported in the publication

1. After i.v. administration in the rat, circa 45% of the administered dose (0.1 mg Ag/kg bw) was detected in the faeces within the first 24h; followed by 18 % in the second 24h period; and then only 4-5% on during d3 and d4 post-dose. A much lesser degree of urinary elimination was found (<0.5% of the total administered dose over the entire 4-day monitoring period).

2. Time-course concentrations of ^{110}Ag determined in plasma and bile up to 2h after i.v. administration of 0.01, 0.03, 0.1, or 0.3 mg Ag/kg bw are presented in Figure 2 of the publication. Peak elimination in the bile occurred 30 min post-dosing. The concentration

[42] Specific activity was unstated.

of Ag in the bile was 16-20 times higher than that of the plasma. Refer to the excerpted Figure:

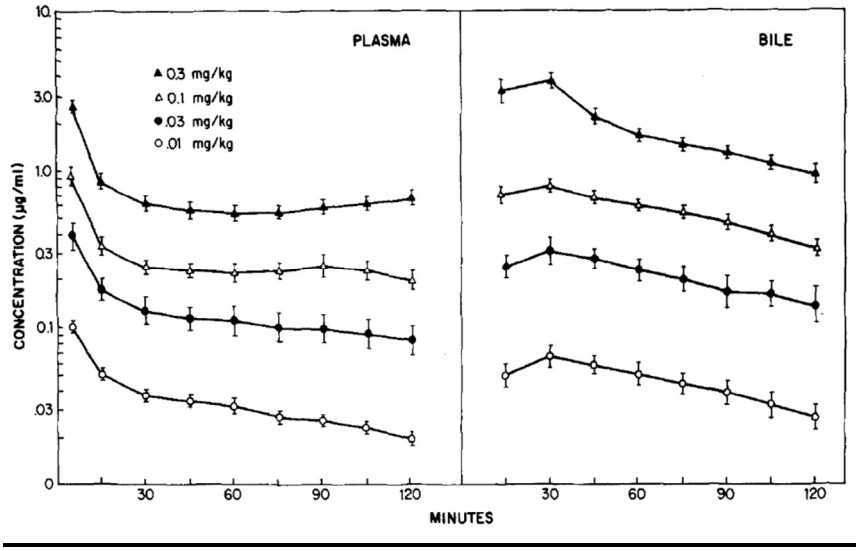


Figure 2. Plasma and bile concentration of ^{110}Ag after various doses of ^{110}Ag were administered i.v. to rats. Each value represents the mean ± SE of 5 to 6 rats.
[*Note: the y-axis is log scale*]

3. Counterpart biliary elimination rate and cumulative biliary elimination time-course plots are provided in Figure 3 of the publication. Dependent on individual dose levels, over the 2h period monitored, 25-45% of the total administered dose of Ag was eliminated in the bile. Refer to the excerpted Figure:

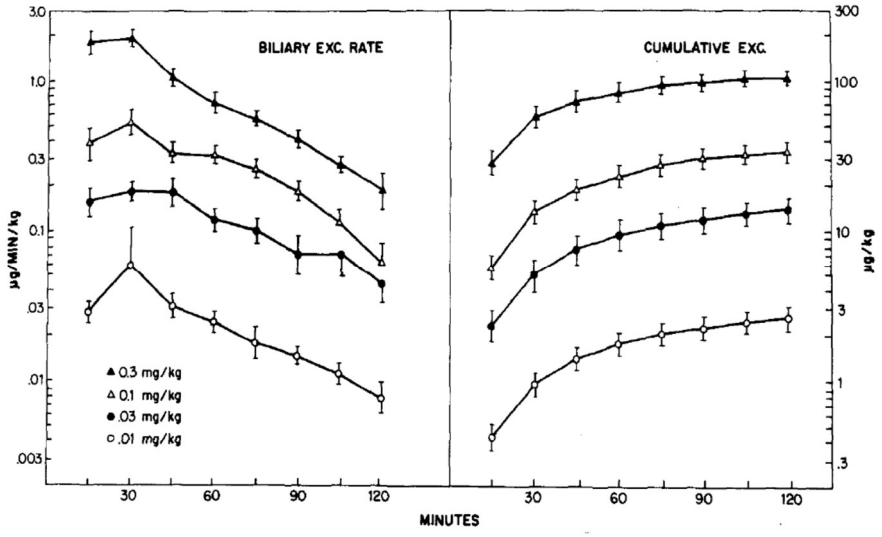


Figure 3. Biliary excretory rate and cumulative excretion of ^{110}Ag after various doses of ^{110}Ag were administered i.v. to rats. Each value represents the mean ± SE of 5 to 6 rats.
[*Note: the y-axis is log scale*]

4. Species variability in the pattern of biliary elimination is addressed in Figure 6 of the publication (via the parameters of plasma concentration; biliary elimination rate; and cumulative biliary elimination). At the 30-min time interval after a dose of 0.1 mg Ag/kg bw, the group mean biliary elimination for rats was 0.25 µg/min/kg; 0.05 µg/min/kg in the rabbit; and 0.005 µg/min/kg for dogs, which had a close correspondence with the rank order of the achieved plasma Ag levels in the three species. It was also inferred by the author that the volume of distribution in the dog (V_d) was substantially higher in the dog than in rats (or rabbits).

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0-t)}$ and estimates of total systemic exposure.

2. As the study design was based on i.v. administration it cannot inform on the degree of biliary elimination occurring following absorption via the oral route (nor the percent absorption via that route).

3. Whilst the relevant data are not detailed in the publication, it is reported that in short-term monitoring (up to 2h post-dose) rats receiving Ag via the i.v. route showed disparities between volumetric plasma and whole blood Ag concentrations. The mean value for the latter group being 53% of that relative to the plasma value immediately post-dose, and then 65% of the measured plasma value by the 2h timepoint. This is notable, and the most likely explanation is that partitioning of Ag to the cellular elements of the blood occurs more slowly (though the full kinetics were not investigated in this study).

4. Based on achieved plasma concentrations in the rat, rabbit and dog (see previous section), it can be assumed that for a given dose of $AgNO_3$ systemic exposure would be higher in the rat than the non-rodent species which were investigated (rabbit and dog).

5. The terminal tissue distribution dataset is largely in accord with later reports related to Ag (elemental and ionic forms) administered parenterally to rats, with relatively greater amounts of Ag detected in the liver versus other organs. Given the low doses of ionic Ag selected and the short period (2h) post-dosing prior to sacrifice in this investigation, other published studies of distribution patterns are more informative in respect of the Ag exposure scenarios of interest. The quite high levels of Ag associated with the brain and testes were determined at only 2 h post-dose, at which point the associated blood compartments of the organs would have contained a high fraction of Ag.

(C) Relevance

1. This investigation is significant in clearly demonstrating that for an ionic form of Ag (administered via the i.v. route) the mode of elimination is almost entirely via the biliary route with only minor amounts eliminated via the renal route. This was confirmed as a consistent pattern in 3 species, viz. rats, rabbits and dogs. Hence the investigation extends first indications from an earlier study (Furchner et al., 1968) that the biliary route is the major pathway by which Ag is eliminated from the body. For example, for rats in this study ~70% of the administered dose was eliminated via this route versus <1% via the renal route[43]. However, the measured rate of biliary elimination showed marked species variation, with the highest rate evident for rats: in relative terms, rat (1); rabbit (0.1) and dog (0.01), with the difference being attributed to differences in Ag concentration in the bile rather than other factors.

[43] Very similar findings on the proportionality of biliary:renal elimination and on biliary elimination kinetics were later obtained in another i.v. route study using $^{110m}AgNO_3$, in which a more extended time-course up to 24h was investigated [Tichý P et al. (1986) Biliary excretion of ^{110m}Ag and its kinetics in the isolated perfused liver in rats. J Hyg Epidemiol Microbiol Immunol. 30: 145-148].

2. The study has negligible relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for a soluble Ag form ($AgNO_3$).

3. The study is one of the few available which directly measure Ag biliary excretion. **Study rank for this purpose: KEY.**

4. Newer publications, including repeat dose regimens, have provided more definitive data on tissue distribution. **Study rank for this purpose: SUPPORTING.**

5. Beyond defining the fundamental modes of elimination relevant to ionic forms of Ag, the TK results have only limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design.

(D) Study robustness and scientific reliability indicators

1. The study was performed prior to GLP and the existence of best practice codification and guidelines for TK studies. Reporting of standard quality and control criteria is absent, as are details of the analytical detection procedure. Therefore, a full evaluation of study robustness is precluded. However, the author and originating institution are well-known with excellent academic credentials, and significant experience of performing distribution and elimination studies with metals/radionuclides. Therefore, it is considered likely that the experiments were diligently designed and performed to reasonable scientific standards.

2. The nature of the vehicle used for i.v. administration is unverified but is assumed to be water rather than isotonic saline.

3. The report does not confirm the use of individual metabolism cages for urine and faecal collection, nor procedures to prevent cross-contamination between these two excreta. It should be assumed that there was a potential for cross-contamination, though this is unlikely to have significantly affected the outcomes of the study.

4. Group sizes for experiments with rats were considered to be adequate for performance of a TK study.

5. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

6. The study was non-GLP.

Gregus and Klaassen, 1979

Test article: Silver nitrate ($^{110m}AgNO_3$)

Species: Rat Strain: Sprague Dawley

Single sex only (males)

Age: unstated; inferred to be young adult animals.

Bodyweight (at commencement of dosing): Males 200 - 300 g.

Intravenous (bolus; via saphenous vein) Single dose

$^{110m}AgNO_3$ as Ag equivalent doses of 0.01; 0.03; 0.1; 0.3 mg Ag/kg bw.

Other non-ferrous and ferrous metal radionuclides were included as comparators (refer to the publication for additional details of the selected metals, their respective dosing regimens and other experimental details).

Ag levels in faeces and urine

TK determinations: urine and faeces collection (24 h period) conducted serially up to 4 days post-dose.

Ag levels in bile and blood

TK determinations: Animals were cannulated via the femoral artery for administration of Ag, and for the serial collection of bile samples at intervals up to 2h post-dose.

Terminal Ag tissue levels

Terminal tissue samples were obtained from rats at 2h post-dose. The tissues evaluated using a scintallography technique were comprised of the liver, kidney, spleen, heart, brain, bone (tibia), stomach, testes, muscle (soleus/gastrocnemius), lung, pancreas, intestine (specific location was not further defined) and blood (whole blood and also plasma).

Ag digestion/analysis methodology:

Digestion was not required (gamma spectrometry was the detection technique).

Precise analytical methodology details are omitted from the publication.

(A) TK data as reported in the publication

1. According to data presented in Table 3 of the publication, Ag ranked in the group of metals most rapidly eliminated (via biliary excretion). Refer to the excerpted Table:

Metal	Dosage, iv[a] (mg/kg)	Fecal excretion (% of dose) of metals				
		Days 1–4	Day 1	Day 2	Day 3	Day 4
Silver	0.1	72.0 ± 9.9[b]	44.1 ± 5.3	18.4 ± 4.6	5.13 ± 0.60	4.32 ± 0.26
Manganese	3	70.0 ± 4.3	50.6 ± 2.4	17.4 ± 2.3	1.32 ± 0.05	0.68 ± 0.02
Copper	1	38.3 ± 3.5	21.8 ± 2.5	16.4 ± 1.1	n.d.	n.d.
Thallium	10	34.4 ± 2.6	14.3 ± 1.3	7.92 ± 0.71	7.39 ± 0.50	7.84 ± 0.39
Lead	3	34.2 ± 2.4	21.8 ± 1.5	8.90 ± 0.49	2.14 ± 0.18	1.34 ± 0.10
Zinc	1	30.6 ± 1.6	11.3 ± 0.80	10.6 ± 0.90	4.20 ± 0.11	4.50 ± 0.10
Bismuth	3	17.1 ± 1.7	12.4 ± 0.56	3.20 ± 0.30	1.10 ± 0.10	0.40 ± 0.07
Cadmium	1	16.5 ± 1.2	9.66 ± 0.69	4.82 ± 0.23	1.37 ± 0.11	0.61 ± 0.06
Mercury	1	15.2 ± 2.4	3.49 ± 0.71	2.63 ± 0.37	5.70 ± 0.49	3.41 ± 0.69
Cobalt	0.3	15.1 ± 2.6	10.7 ± 1.7	2.63 ± 0.46	1.00 ± 0.17	0.67 ± 0.13
Cesium	30	11.2 ± 2.2	2.89 ± 0.57	3.40 ± 0.59	3.16 ± 0.58	1.78 ± 0.45
Gold	1	9.80 ± 0.48	3.20 ± 0.10	2.90 ± 0.10	2.30 ± 0.10	1.40 ± 0.12
Iron	0.3	8.25 ± 0.60	1.80 ± 0.20	2.80 ± 0.23	2.13 ± 0.07	1.53 ± 0.10
Arsenic	1	6.03 ± 0.18	5.00 ± 0.04	0.71 ± 0.08	0.21 ± 0.02	0.11 ± 0.02
Methyl mercury	1	5.60 ± 0.94	1.83 ± 0.28	2.49 ± 0.32	0.78 ± 0.09	0.51 ± 0.14
Chromium	3	5.23 ± 1.2	2.90 ± 1.16	1.16 ± 0.23	0.70 ± 0.07	0.46 ± 0.07
Selenium	0.3	4.34 ± 0.89	2.36 ± 0.90	0.93 ± 0.10	0.63 ± 0.10	0.40 ± 0.07
Tin	1	1.17 ± 0.18	0.19 ± 0.04	0.39 ± 0.03	0.36 ± 0.07	0.21 ± 0.03

Table 3. Fecal excretion of metals in rats. Dosages refer to metal ions. Means ± SE of 4 to 6 rats. n.d. = non-detectable.

2. In contrast, Ag elimination via the renal route comprised <0.5% of the administered dose over a 4-day period (Table 4 of the publication).

3. Percentage biliary elimination values for the various doses of $^{110m}AgNO_3$ administered via the i.v. route were presented in Table 5 of the publication.

4. For the various metals which were included in the study, Table 8 summarised the values for: (i) percent elimination [total/faecal/urinary]; (ii) percentage biliary elimination by 2h; (ii) organ Ag distribution (for liver, kidney and intestine). Refer to the excerpted Table:

Metal	Dosage, iv[a] (mg/kg)	Excretion (% of dose in 4 days)			Biliary excretion[c] (% of dose in 2 hr)	Organ distribution[d] (% of dose in organ)		
		Total[b]	Fecal	Urinary		Liver	Kidneys	Intestine
Silver	0.1	72.3[e]	72.0	0.30	34.5	43.4	0.75	3.02
Manganese	3	70.0	70.0	0.05	25.6	22.7	2.63	n.d.
Copper	1	46.1	38.3	7.92	5.11	41.3	7.35	5.65
Thallium	10	44.6	34.4	10.2	0.16	7.63	5.40	8.74
Lead	3	37.8	34.2	3.63	3.88	43.4	4.29	3.86
Zinc	1	31.6	30.6	1.00	0.35	12.0	2.62	3.86
Cadmium	1	16.5	16.5	0.02	3.98	66.9	1.96	8.90
Iron	0.3	8.39	8.25	0.14	0.23	30.4	2.49	3.64
Methyl mercury	1	6.12	5.60	0.52	0.71	10.5	3.93	5.26
Cobalt	0.3	87.7	15.1	72.6	3.39	22.8	10.2	3.16
Cesium	30	37.7	11.2	26.5	0.34	14.7	4.29	15.1
Gold	1	31.9	9.80	22.1	0.38	9.66	2.63	1.11
Selenium	0.3	21.9	4.34	17.6	4.65	39.9	3.82	2.41
Chromium	3	21.5	5.23	16.3	0.05	20.7	12.9	1.23
Bismuth	3	38.3	17.1	21.2	6.27	5.78	18.6	13.2
Mercury	1	31.5	15.2	16.3	0.60	11.7	31.2	4.68
Arsenic	1	16.9	6.03	10.9	24.1	4.10	2.01	1.43
Tin	1	2.78	1.17	1.61	0.02	72.8	0.56	0.40

Table 8. Excretion of metals and their distribution to excretory organs. Dosages refer to metal ions. Total excretion is the sum of fecal and urinary excretion. Means ± SE of 4 to 6 rats. n.d. = not determined.

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0-t)}$ and estimates of total systemic exposure.

2. As the study design was based on i.v. administration it cannot inform on the degree of biliary elimination occurring following absorption via the oral route (nor the percent absorption via that route).

3. Percentage biliary elimination values for doses of between 0.01 – 0.3 mg Ag/kg bw (as $^{110m}AgNO_3$) via the i.v. route were consistent with those previously reported in Klaassen, 1979. The same was true of tissue distribution patterns measured 2h post-dose.

(C) Relevance

1. This investigation reconfirms other reports of the importance of biliary elimination in the case of an ionic Ag reference compound ($AgNO_3$) – refer also to commentary on Klaassen, 1979 (as further confirmed in the report by Tichý et al., 1986). The study is one of the few available which directly measured Ag biliary excretion. **Study rank for this purpose: KEY.** In comparative work with other metals, it was established that Ag resides in the group of metals which are principally cleared via biliary rather than a urinary mode of elimination: inter alia, lead, zinc, manganese, copper, cadmium and iron. Of the other metals studied, manganese had the most similar elimination profile and kinetics to Ag.

2. The study has negligible relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for a soluble Ag form ($AgNO_3$).

3. Newer publications, including repeat dose regimens, have since provided more definitive data on tissue distribution. **Study rank for this purpose: SUPPORTING.**

4. Beyond defining the fundamental modes of elimination relevant to ionic forms of Ag, the TK results have only limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design.

(D) Study robustness and scientific reliability indicators

1. Reporting of standard quality and control criteria is absent, as are details of the analytical detection procedure. Therefore, a full evaluation of study robustness is precluded. However, the authors and originating institution are well-known with excellent academic credentials, and significant experience of performing distribution and elimination studies with metals/radionuclides. Therefore, it is considered likely that the experiments were diligently designed and performed to reasonable scientific standards.

2. The vehicle used for i.v. administration was appropriate in the case of Ag compounds (water was utilised rather than isotonic saline).

3. Individual metabolism cages were used for urine and faecal collection, although procedures to prevent cross-contamination between these two excreta were not described. It should be assumed that there was a potential for cross-contamination, though this is unlikely to have significantly affected the outcomes of the study.

4. Group sizes for experiments with rats were considered to be adequate for performance of a TK study.

5. Overt toxicity considered sufficient to interfere with TK outcomes was not apparent.

6. The study was non-GLP.

Park, 2013 (Single-dose study)

Test article: **Ionic Ag reference: Ag in 2% HNO_3 [10 mg Ag/mL]** – hereafter referred to as "Ag^+"
It is inferred this test article corresponded to $AgNO_3$ in solution, and that Ag equivalent values can be related to the stated Ag content.

Silver NP (8 nm); citrate-capped[44] (OECD reference nanoform)

Species: Rat **Strain: Sprague Dawley**

Single sex only (males).

Age: 8 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): 225 g.

Oral (gavage) **Single dose**

Ag^+: 2; 20 mg/kg bw **2; 20 mg/kg bw as Ag equiv.**

AgNP: 2; 20 mg/kg bw

Ag levels in whole blood (heparinised)

TK determinations: d1. Timepoints: 0, 4, 8, and 24h.

Ag levels in faeces / urine

Faecal and urinary Ag levels via 24 h collection post-dose.

Terminal Ag tissue levels

Terminal tissue samples were obtained 24 h post-dose (comprising a limited tissue set).

Ag digestion/analysis methodology:

Nitric acid/peroxide-microwave digestion / ICP-MS.

(A) TK data as reported in the publication

1. Blood concentration of Ag following oral administration are reported as time-course data in Figure 2 of the publication. Data relating to systemic availability parameters are summarised as follows, together with additional inter-treatment comparator indices:

	Ag^+		AgNP	
	2 mg/kg bw	20 mg/kg bw	2 mg/kg bw	20 mg/kg bw
C_{max} (ng/mL)	90	200	60	80
t_{max} (h)	7	14	12	13
$AUC_{(0-t)}$ (ng.h/mL)	45120	91440	29280	37920
C_{max} /D	45	10	30	4
$AUC_{(0-t)}$ /D	22560	4570	14640	1900
Relative $AUC_{(0-t)}$ Ag^+: AgNP	**1.5**	**2.4**		

AUC units adjusted ng.h basis. Appropriate rounding has been applied to TK value conversions. The published TK data were derived using BACalc, 2007, ver. 1.0.0; Korean FDA.

[44] The AgNP was the same as that selected in an earlier study by this laboratory: see Park et al., 2011 (which is evaluated elsewhere in this document).

2. Terminal Ag in tissue levels are reported in Figure 3 of the publication.

3. Cumulative urinary and faecal Ag content values (to 24 h) are provided in Figure 4 of the publication.

(B) TK data assessment and analysis

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = MEDIUM (raw data unavailable; interpolations made from the publication figures). In any inter-study comparison, it should be borne in mind that all values are derived from these investigations relate only to male animals.

1. Inter-study comparison of derived systemic exposure parameters for the AgNP dosed groups to those in a previous investigation by this laboratory (which relied on the same AgNP test article, and an equivalent dosing regimen and similar dose levels [1 or 10 mg/kg bw]) revealed both congruent and non-congruent values. Refer also to the assessment of the other publication (Park et al., 2011) in this report. For the high-dose groups of both studies, the $AUC_{(0-t)}$ /D values were very similar. However, in the case of the low-dose groups the $AUC_{(0-t)}$ /D was more than 10-fold greater in the case of Park (2013) than that evident in Park et al. (2011) at 14640 ng.h/mL versus 1160 ng.h/mL, respectively. However, AgNP inter-group C_{max} and t_{max} values showed a higher degree of internal consistency in Park, 2013 with those in Park et al., 2011. Examination of the time-course data also shows disparities in shape of the blood concentration-time curves (up to 24 h) between the two studies, especially when the respective high-dose AgNP groups are considered. Overall, a basis for these discrepancies in systemic exposure parameters is unclear. See also remarks in Study Robustness section.

2. As stated by the report's author, the results suggest relatively poorer absorption of a small citrate-capped AgNP (8 nm) via the oral route compared to an ionic Ag form (at the same nominal Ag equivalent doses. In terms of relative $AUC_{(0-t)}$ indices, the systemic exposure in the case of the Ag^+ test article was either 1.5 or 2.4-fold higher, for the groups receiving either 2 or 20 mg/kg bw, respectively; which represent quite modest differences. It should be noted that in the case of this particular investigation, there are several study robustness considerations which limit interpretative Confidence level; for example, uncertainties relating to the presence of coexistent ionic Ag content of the AgNP test material, and in the blood sampling methodology used.

3. In the absence of parallel parenteral route dose groups, it is not possible to derive an absolute bioavailability (F) value for via the oral route for the ionic Ag and AgNP test articles.

4. The dataset for terminal Ag in tissue concentrations covers only 3 tissues (viz. liver, kidney and lung) which limits the value of the study in respect of elucidating the full pattern of Ag distribution. Pairwise comparisons between Ag^+ and AgNP-treated groups lend support for an inference that greater absorption of Ag occurred in the case of the ionic Ag test article, although the differences were not statistically significant in all cases. Ag tissue levels in the liver were higher than in the lung or kidney; but compared to most other published short-term tissue distribution studies on AgNP and ionic Ag, the relative concentration difference was not as marked between the liver and the two other tissues.

5. The urinary and faecal Ag datasets are quite basic, e.g. it is not possible to calculate the percentage of administered dose eliminated via each mode. However, in keeping with

many other previous Ag TK investigations, the measured Ag content in the urine was minimal compared to faecal levels.

6. For rats which received AgNP, the faecal Ag content did not show a linear relationship in respect to dose when the 2 and 20 mg/kg bw groups were compared (the latter group apparently exhibiting a 20-fold higher extent of elimination by this mode). Why this should be the case is not obvious. Not all ingested Ag would be expected to be voided within 24 h which may impact the lower dose level to a greater extent.

7. Group mean faecal Ag content in ionic Ag-treated rats was lower than AgNP groups when equivalent dose levels were compared, i.e. an approximately 2.5-fold lower at 2 mg/kg bw Ag^+, whereas at 20 mg/kg bw Ag^+ there was a much greater 125-fold difference. The high-dose group value being skewed by the effect discussed in Point 6 above. One possible inference is that a relatively high proportion of the administered dose was absorbed from the gut in the case of ionic Ag and the lower dose of AgNP versus the situation for the higher AgNP dose (suggesting a change to zero-order kinetics for the latter).

(C) Relevance

1. The study is relevant to determination of basic TK parameters regarding systemic exposure via the oral route for a form of elemental Ag (a single type of AgNP) and an ionic Ag test material ($AgNO_3$ in solution). **Study rank for this purpose: SUPPORTING (study robustness considerations preclude its ranking as a key study)**.

2. There are indications that a moderately higher systemic exposure of the test species was obtained with ionic Ag versus the selected AgNP (8 nm; citrate-capped). The absence of ionic Ag quantitation data for the AgNP test article reduces the Confidence level attributable associated with this finding.

3. The publication also provides limited supporting information on Ag elimination and distribution, although superior TK studies exist for these aspects.

4. The investigation has limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. The TK study was performed in the same species (rat), albeit in male animals only; via a relevant route and dosing regimen (incorporating the oral route); and it includes ionic Ag dose levels which align with those of interest[45]. However, the design was based on a single dose rather than repeated dosing and steady-state TK conditions are not expected to have been achieved.

5. The absence of certain key procedural details, and certain anomalies in its reporting, represent an impediment to a superior KL ranking for this study. Refer also to the section 'Study robustness indicators'.

[45] Sprando et al./Babu et al.: Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

(D) Study robustness and scientific reliability indicators

1. The selected AgNP (8 nm; citrate-capped) test article is a well characterised OECD reference material. The AgNP characterisation procedures listed were evaluated as robust for physical parameters (also taking into account the use of an OECD reference material). No quantitation of Ag present in the ionic form in the AgNP stock suspensions was described, which is sub-optimal in terms of robustness. The use of an OECD reference nanoform partly mitigates this concern, but interpretation of study outcomes is complicated by the absence of concurrent ionic Ag measurements.

2. Concentration verification for dosing solutions was not reported as performed. No detailed descriptions of the procedures / vehicles used for gavage are provided in the publication.

3. The methods section of the paper makes clear reference to collection of serial blood samples from the jugular vein of animals at timepoints of 0, 4, 8 and 24 h. However, the legend in Figure 2 covering the Ag in blood time-course graphs states that the data relates to blood collection from the "caudal vena cava" (which would infer terminal sampling from separate animals) at these timepoints. Methodological descriptions are insufficient to be completely certain which is correct, but it is considered more likely that serial sampling was the approach taken. This discrepancy does impact on confidence level in the report and its robustness in relation to the key systemic exposure data. The serial blood sample volumes collected from the animals (0.5 mL at each timepoint) were relatively large. The cumulative blood volume prior to the terminal (24 h) final sample point at 1.5 mL corresponds to an amount which could have a borderline influence on certain TK parameters. However, it is considered unlikely that the magnitude of any impact would be major.

4. The group sizes were considered to be adequate for performance of a TK study.

5. For Ag concentration determinations, the preparative microwave-based technique involved digestion with hydrogen peroxide/nitric acid mixture rather than nitric acid alone, which is a less commonly used technique. Data from internal standards and recovery values were not provided. However, the reported LOD via ICP-MS analysis showed high sensitivity (<0.001 ppb).

6. Urine and faecal samples were obtained via the use of individual metabolic cages in a manner which minimised the potential for contamination.

7. Some toxicity was reported following AgNP and $AgNO_3$ treatment, including slight elevations in serum transaminase levels, and in the case of $AgNO_3$, hepatic inflammatory change and hepatocellular necrosis. Whilst the liver has been shown to be a target organ in Ag toxicity, this typically has not been reported in the rat after equivalently low doses (also keeping in mind that only a single p.o. dose was administered). From the description of the findings there are reasons to doubt the robustness of the histopathology assessment (e.g. severity gradings were omitted). On balance, it is considered that the limited toxicity which was evident is unlikely to have confounded TK outcomes.

8. The study was non-GLP.

Bachler et al., 2013 (PBTK model application)

Physiologically based toxicokinetic model (PBTK)

The basic PBTK model corresponds to WHO IPCS guidelines[46] and their associated general parameter assumptions. Membrane-limited model. An assumption of first-order (concentration-dependent) kinetics was made. Silver-specific TK values were built into the model. Two separate sub-models were incorporated to cover: (A) ionic Ag; and (B) AgNP.

Data from TK studies covering the following Ag forms were input into the PBTK model:-

$AgNO_3$ / AgAc / Ionic silver solutions

Used in sub-model A (defined below), i.e. readily soluble Ag forms. Refer to the original TK publications for further details of test articles.

AgNP (various: 15 – 150 nm)

Used in sub-model B (defined below). Refer to the original TK publications for further details of test articles.

Species: Rat **Strain:** refer to the original TK publications.

Sex / Age / Bodyweight: refer to the original TK publications. Mean bodyweight in the PBTK model was calibrated to an average of that in the in vivo rat TK studies (viz. 300 g.).

In vivo study exposure route used for <u>model building</u>: i.v. only

TK data for extravascular administrated nanoAg (e.g., oral, subcutaneous, and i.p. routes) were not considered.

In vivo TK studies used for <u>model building</u>:

Sub-model A (ionic Ag) - Klaassen (1979) Biliary excretion of silver in the rat, rabbit, and dog. Toxicol Appl Pharmacol. 50: 49-55.

Sub-model B (AgNP) - Lankveld et al., (2010) The kinetics of the tissue distribution of silver nanoparticles of different sizes. Biomaterials 31: 8350-8361.

Note: Data from human TK investigations were also input into the model. The reader is referred to the publication itself for these specifics.

Input TK reference data: Distribution, metabolism (*sic.*) and elimination parameters as previously established in the various in vivo TK investigations.

In vivo TK studies used for <u>model verification</u> (both sub-models):

These studies included, but were not limited, to the following:

Kim et al. (2008) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol. 20: 575–583.

Loeschner et al. (2011) Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part Fibre Toxicol. 8: 18.

Park et al. (2011) Bioavailability and toxicokinetics of citrate-coated silver nanoparticles in rats. Arch Pharm Res. 34: 153–158.

Furchner et al. (1968). Comparative metabolism of radionuclides in mammals-IV. Retention of silver-110m in the mouse, rat, monkey, and dog. Health Phys. 15: 505–514.

[46] WHO IPCS Harmonisation Project document series No. 9: Guidance on principles of characterizing and applying PBPK models in risk assessment. International Programme on Chemical Safety; 2010.

(A) TK data as reported in the publication

The reader is referred to the first parts of the publication covering specifics of the PBTK model in respect of absorption, distribution, 'metabolism' (in actuality physiologically-relevant complexation of Ag), and excretion (elimination) – see also the next section '(B) TK data assessment and analysis'. Selected results arising from the application of the model are summarised below.

1. The PBTK sub-models were validated against published TK data for ionic silver and nanoAg in rats (and humans). Absorption fractions and rates were fitted to the respective data sets, so the absorption part of the models was not validated, but only the distribution, "metabolism" (actually complexation processes), and elimination parts of the models. Determined absorption, distribution, metabolism (*sic.*) and elimination rates, are also further detailed in a Supplementary Information paper (www.research-collection.ethz.ch/handle/20.500.11850/153962). This includes the derivation basis for the intestinal absorption fractions.

2. Actual and model-predicted Ag tissue levels were compared from two repeat dose (28-day) oral route studies[47] in Figure 4 of the publication. In general, there was good correspondence between the actual in vivo data for both the AgNP forms and for the ionic reference substance (AgAc) and those of the model-predicted tissue exposures (refer to the excerpted Figure):

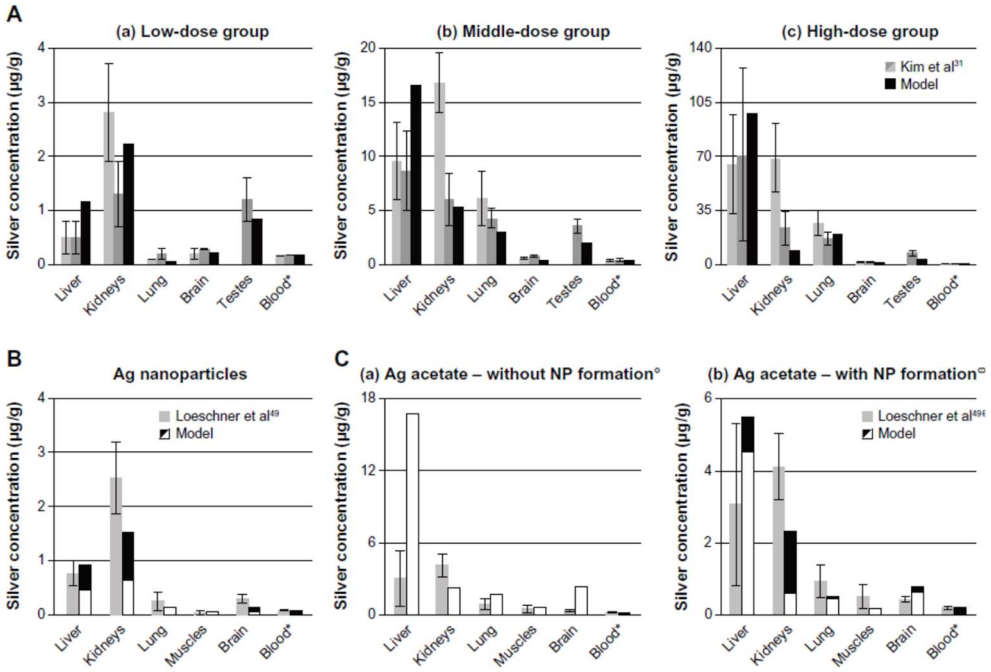


Figure 4 Comparison of the PBPK model simulations to toxicokinetic data of rats after oral exposure for 28 days. Notes: (A) 30 (a), 300 (b), and 1000 (c) mg/kg/day 60 (52.7–70.9) nm particles (B) 12.6 mg/kg/day 14 ± 2 nm particles (11% ionic fraction), and (C) 9 mg/kg/day silver acetate without (a) and with (b) consideration of the formation of nanoparticles in the GI-tract. Light grey: female rats; dark grey: male rats; black: nanosilver; white: ionic silver; *intestinal absorption fractions were fitted to match blood silver levels (listed in Table S7); °transformation of soluble silver salts to silver nanoparticles within the digestive tract. Error bars represent standard deviations; n = 5 to n = 10.

[47] Refer to separate commentary in this report on the studies by Kim et al. (2008) and Loeschner et al. (2011).

3. When parameters for ionic Ag were calculated in a simulation which took account of secondary AgNP formation in the gut (assumed as 33% ionic Ag and 67% nanosilver), the model outputs then more closely resembled the actual in vivo data (see publication Figure 4C; “with NP formation”).

4. Modelled elimination rates matched in vivo data from rats which received 1 mg/kg AgNP (8 nm) in Park et al., 2011. The group mean values reported by Park et al. were 9.0% and 0.08% for faecal and urinary modes, respectively, whereas the model predicted 13.0% and 0.18%, respectively, for biliary and urinary modes.

(B) TK data assessment and analysis

This investigation was not a conventional TK study, but instead was based on a PBTK model for ionic Ag and AgNP whereby certain in vivo TK experiments are primarily used for model-building purposes. Hence it is not considered appropriate to make the standard judgement on Confidence level for systemic exposure parameters. Conclusions derived from the model on human TK are not considered in detail here (the reader should refer to the original publication; instead emphasis is given to outcomes from use of the model which inform on TK in experimental species (particularly that of rodents).

1. The aim of the study was to build and validate a PBTK model which comprised of two sub-models, one for ionic Ag and one for AgNP in order to predict tissue distribution and achieved tissue concentrations following exposure to Ag. Schematics of the connectivity between the rat-based model and the human predictive model is provided in Figure 1 of the publication, with the compartments and exposure routes detailed in Figure 2A and B (refer to excepted Figures):

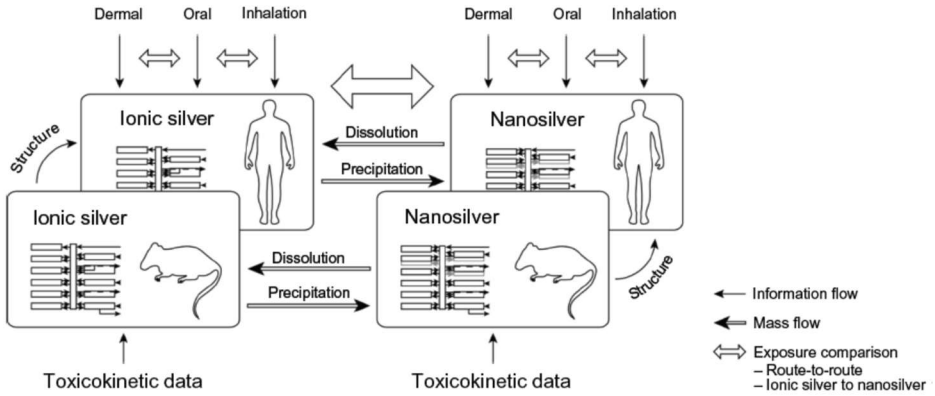


Figure 1. Basic concept of couplings and dependencies between the different PBPK models.

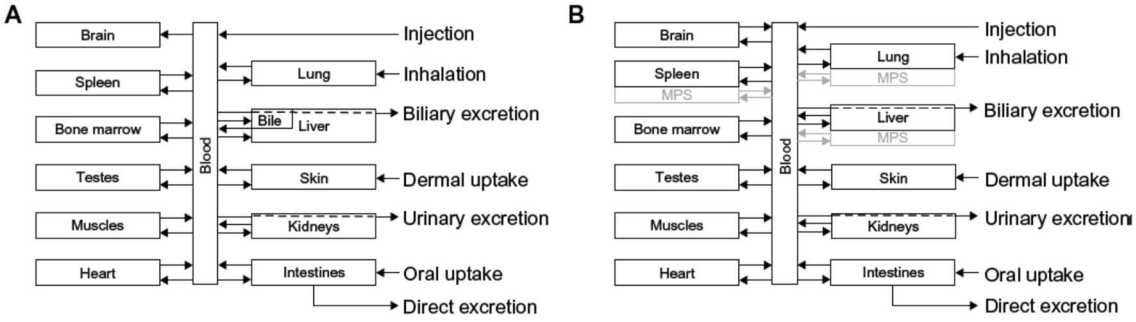


Figure 2. Schematic diagram of the PBPK model structures for (A) ionic and (B) nanoparticulate silver, which were used both for rats and humans. Note: In the ionic silver model no transport of silver from the brain to the blood was modelled. Note: Model parameter information and determined TK values are published separately from the main paper as supplementary information (www.research-collection.ethz.ch/handle/20.500.11850/153962).

Comments on the applicability and robustness of the PBTK model are set out in the section on study robustness.

2. Intestinal absorption fractions derived from the dataset published in Kim et al. (2008) for AgNP are non-linear, with the fraction diminishing with increasing dose (30; 100 or 1000 mg Ag/kg bw/d). Comparison of these calculated fractional absorption values with those for the AgNP form studied by Loeschner et al. (2011) were similar when dose-levels are matched, i.e. circa 0.9% absorption. For the ionic Ag form in the Loeschner et al. study the calculated fractional absorption value is 3 to 4-fold higher (dependent on whether secondary NP formation is simulated, or not).

3. An assumption made by the authors was that the gender-related differences in rodent Ag TK studies have been minimal. Therefore, they did not incorporate gender-specific kinetic adjustments in their model. However, the current state of knowledge is that sex-related differences may be more significant than was thought at the time, particularly in respect of systemic exposure (as well as a few tissue distribution pattern differences).

4. The model apparently accommodated various sizes of AgNP; tissue distribution being acceptably predicted for 15, 20, 60, 80, and 110 nm sized NP. The applicability domain for the AgNP sub-model for NP size was set as between 15 – 150 nm due to some reservations by the authors about renal filtration thresholds relating to smaller AgNP. Based on the elimination phase dataset which has been established from in vivo studies, it is probable that the model would also have reasonable applicability to slightly smaller AgNP; for example, in the 5 – 15 nm range.

5. The principal oral route studies used for experimental validation of the model outputs for AgNP rely on AgNP with two different stabilisation/dispersion systems, viz. a PVP-capped NP and an uncapped CMC-formulated NP. This apparently did not substantively influence the outcomes in respect of Ag quantitative distribution predictions. More confidence could be attached to the inference about the limited influence of NP coating if further in vivo studies had been used for validation with additional AgNP coating types, and if multifactorial aspects had been considered (e.g. the route of administration). A more recent study via the i.v. route in mice has reported some variation in tissue distribution based on AgNP coating type[48].

6. Useful TK modelling results for Ag in humans, and their application to risk assessment, were also presented in the paper; but are not evaluated further herein as they are outside of the scope of this review.

(C) Relevance

The study is relevant to determination of basic TK parameters (absorption, distribution, elimination) connected to systemic exposure via the oral route for elemental Ag (several types of AgNP) and for ionic Ag forms. In addition, there is consideration of Ag complexation effects versus TK impacts, which is not commonly evaluated/modelled. **Study rank for this purpose: SUPPORTING**.

1. The high-level relevance of the model relates to the prediction of tissue distribution and achieved tissue concentrations in respect of more water-soluble ionic Ag forms and also AgNP, as well as tissue complexation/fixation and elimination phases. As the authors

[48] Pang et al. (2016).

stated, the absorption phase of the models was not separately validated. In these TK phases, the PBTK produced reasonable predictions versus in vivo TK values obtained via various routes for both AgNP and ionic Ag in rats. A later publication (Juling et al., 2016) re-visited oral absorption considerations. It should be noted that due to the date of the study, a raft of more recently published TK data on Ag (including ionic forms) was not available to the investigators – this was partly mitigated by the follow up publication (Juling et al., 2016), but data from studies subsequent to this obviously have not been considered in respect of model verification and extension.

2. The investigation has relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design (particularly the latter). It may be possible to use such a PBTK model to predict the tissue exposures achieved in reproductive studies on various Ag substances, including AgAc.

3. The findings of this work for are consistent with a lower estimated oral bioavailability for AgNP versus ionic Ag. For AgNP the modelled bioavailability seems to be non-linear with dose, but approximates to 1% as a worst case for the NP-size applicability domain defined. This is less than some estimates derived from some in vivo work (see commentary in the Conclusions section related to oral bioavailability parameters). For one ionic reference form (AgAc), the corresponding value is predicted to be between 3.25% and 3.85% of administered dose via the oral route. It is important to note that only two oral route TK studies were relied on for model verification, which is not optimal.

4. The study confirms findings elsewhere that a PBTK model of administered ionic Ag TK should take account of the de novo formation of secondary NP within the body, e.g. in the GI tract.

5. The authors conclude that the size of AgNP (at least for NP above 15 nm) was not a major determinant of tissue distribution. Based on other conventional TK reports, e.g. that larger particles are preferentially accumulated by the spleen, some researchers draw contradictory conclusions (refer to 'Conclusions' sub-section 'Influence of nanoparticle size in AgNP TK').

(D) Study robustness and scientific reliability indicators

1. PBTK model validity:
    a. The core model was specifically adjusted with separate sub-models for ionic Ag and nanoAg (based on data from i.v. route only in vivo TK studies). This is an appropriate basis for a basic validation of the model, although it does not include parameters from extravascular administration routes. Note: the oral route applicability domain was examined in a later study (refer to the separate commentary in this document for Juling et al., 2016).
    b. First-order kinetics (i.e. concentration dependent processes) were assumed. This is likely to be an over-simplification of the actual in vivo TK situation; for instance, there is evidence in other TK studies on Ag of transition to concentration-independent zero-order processes (particularly at high doses). However, such a rationalisation is typically applied in PBTK modelling and it is not considered invalidate the main conclusions from the model.

c. The model incorporated factors related to glutathione (GSH) levels (total body and specific tissue), given the propensity of Ag to interact with S-centres and since GSH is the soluble thiol present at the greatest concentration in various tissue compartments. Due account was also taken of complexation in tissues by sulphur (to form silver sulphide depots) and similar complexation with tissue selenium, although for the latter modelling details are not provided. Overall, all these considerations are considered to be relevant to the Ag distribution phase and tissue kinetics.

d. The membrane-limited model was adjusted for the influence of the blood brain barrier (BBB) and blood testis barrier (BTB), which is an appropriate approach in the case of Ag, where some TK studies have reported differing toxicokinetics for these organs.

e. For the elimination phase, due account was taken of the predominance of biliary elimination versus that of the renal mode.

f. A correction for the formation of secondary AgNP from ionic Ag was applied.

g. Where AgNP study data was used for the in vivo verification dataset, the impact of ionic Ag content could be considered for some <u>but not all</u> studies (e.g. Kim et al. 2008 is does not report this parameter).

Without access to the model itself, it is not possible to verify the TK parameters for Ag which were derived from the model. However, as far as can be determined from the specifics presented in the paper, its validity and applicability domain appear to be acceptable.

2. The experimental in vivo TK studies (which were selected for the purposes of the model TK input reference values) were themselves mainly of reasonable quality. One chosen i.v. reference study, viz. Kim et al., 2008, is considered to have a number of design and robustness limitations (as per the commentary elsewhere in this document). None of the selected studies were conducted to GLP, nor are they categorisable as the highest Klimisch Rank (i.e. KL 1), but this is the case for nearly all the available TK dataset for Ag.

3. GLP status: Not applicable – modelling investigation.

Juling et al., 2016 (Single-dose TK study; with an associated PBTK model)

Test article: Silver NP (16 nm); stabilised with polyoxyethylene-based non-ionic surfactants[49]

The selected AgNP is a BAM[50] reference nanoform

Silver nitrate ($AgNO_3$)

Species: Rat Strain: Wistar (Han)

<u>Single sex</u> only (males).

Age: 9 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): unstated.

Oral (gavage) / Intravenous (bolus; via tail vein) Single dose

Oral: $AgNO_3$ – 31.5 mg/kg bw $AgNO_3$ / 20 mg/kg bw as Ag equiv. AgNP – 20 mg/kg bw.

IV: $AgNO_3$ – 6.3 mg/kg bw $AgNO_3$ / 4 mg/kg bw as Ag equiv. AgNP – 4 mg/kg bw.

Terminal Ag tissue levels

Terminal tissue samples were obtained via serial sacrifice of dosed groups as follows:-

Oral route: d1 (24h post-dose), d2, d3, d7.

i.v. route: d1 (24h post-dose).

Ag levels in faeces

Faecal Ag levels via cumulative 24 h collection post-dose.

Ag digestion/analysis methodology:

Nitric acid/HCl-microwave digestion.

AAS (ion release / tissue concentration verifications); ICP-MS (Ag content of tissues and faeces).

Physiologically based toxicokinetic model (PBTK)

The basic PBTK model corresponds to WHO IPCS guidelines[51] and their associated general parameter assumptions. It was a membrane-limited model. An assumption of first-order (concentration-dependent) kinetics was made. Refer also to the further commentary in this document on an earlier publication relating to the PBTK model (Bachler et al., 2013).

[49] 4% (w/w) polyoxyethylene glycerol trioleate (*Tagat TO*) and 4% (w/w) polyoxyethylene (20) sorbitan monolaurate (*Tween 20*).

[50] German Federal Institute for Materials Research and Testing.

[51] WHO IPCS Harmonisation Project document series No. 9: Guidance on principles of characterizing and applying PBPK models in risk assessment. International Programme on Chemical Safety; 2010.

(A) TK data as reported in the publication

1. Ag contents of tissues (normalised for percentage of the total administered Ag dose) are given in Figure 1A (i.v. route) and 1B (oral route). Refer to the excerpted Figure.

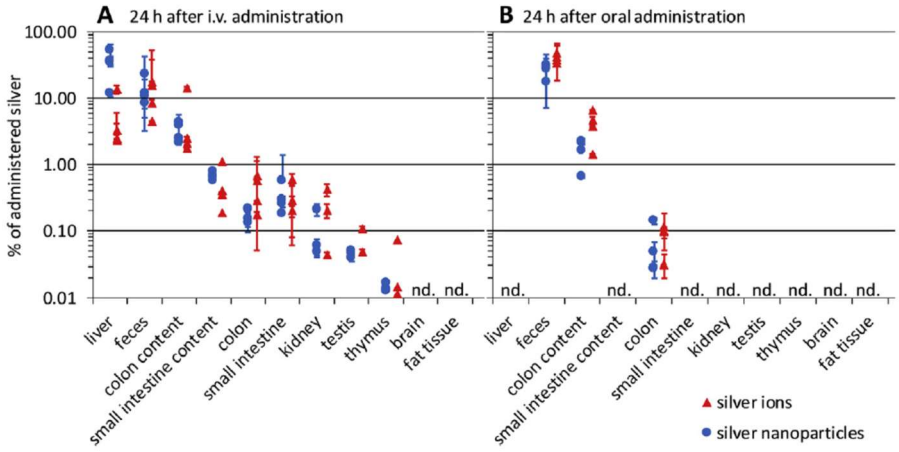


Figure 1. Silver contents of different tissues from rats, presented as percent of the total administered dose of silver. Mean ± standard deviation are shown for each individual animal. (A) Silver content 24 h after intravenous injection of silver nanoparticles (blue circles) or silver ions in the form of silver nitrate (red triangles). (B) Silver content 24 h after oral administration of silver nanoparticles (blue circles) or silver ions in the form of silver nitrate (red triangles). Abbreviation: nd - not detectable.

2. For faecal samples, cumulative silver content from the time of treatment to the time of sampling is provided in Table S3 (Supplementary information publication).

3. Figure 3 A and B in the publication summarises the PBTK model outcomes for AgNP and $AgNO_3$, respectively. Refer to excerpted Figures and also to the commentary in the next section.

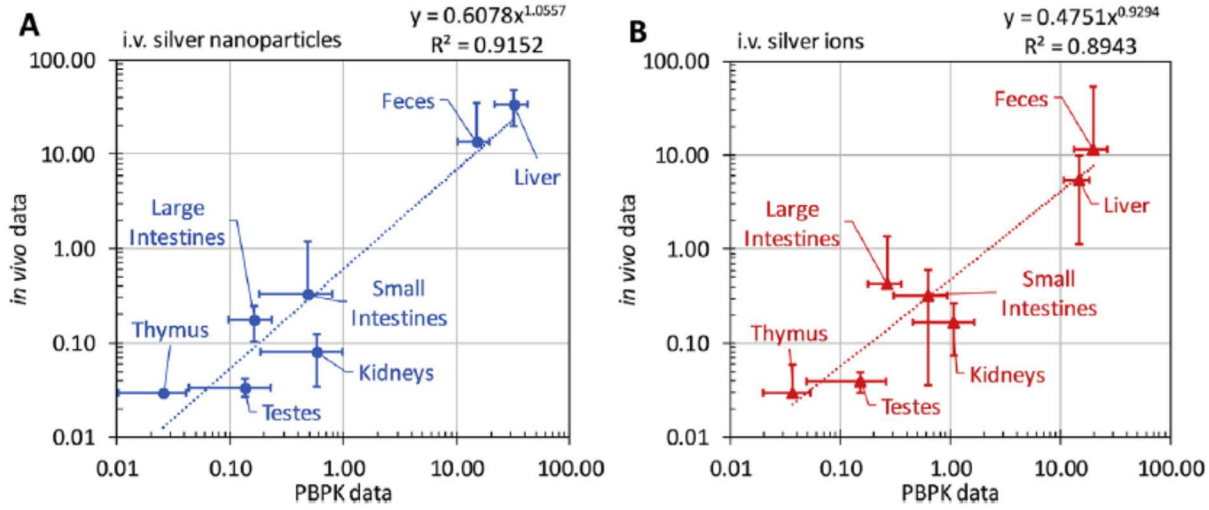


Fig. 3. Toxicokinetic modeling of tissue distribution of silver following intravenous application. (A) Best fit for the comparison of toxicokinetic modeling and in vivo data for intravenous application of silver nanoparticles. (B) Best fit for the comparison of toxicokinetic modeling and in vivo data for intravenous application of ionic silver. Mean ± standard deviation is shown for experimental data.

It may be seen that the fit was good for several tissues including the GI tract and liver, but poorer for the kidney and testes.

4. In terms of Ag speciation, it was stated that the model predicted that following i.v. administration of AgNP, 86% of the Ag was present as large nanoparticles and 14% as ionic Ag. Whereas for ionic silver ($AgNO_3$) an unexpectedly small fraction was predicted to be in ionic form (27%); with most silver predicted to be present as secondary small (56%) or large nanoparticles (17%).

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure by a conventional TK approach.

2. Based on the presented results in Fig. 1, following i.v. administration of either AgNP or $AgNO_3$, at 24 h post-dose the highest levels of Ag were associated with the faeces, GI tract content (particularly colon), with lower amounts present in most other tissues. The Ag distribution was quantitatively comparable for both Ag forms across nearly all tissues. Very low levels (<0.1% of the total administered Ag dose) were detected in the testes, with brain levels being undetectable. Via the oral route Ag was only found to be faecal or GI tract-associated, with non-detectable levels in all other organs. The levels were again comparable between AgNP and the ionic Ag reference.

3. This study provides further confirmation that the absolute oral bioavailability of Ag (either in AgNP or ionic form) is low, but the in vivo data do not support a quantitative bioavailability calculation. Autometallography staining demonstrated that the portion of the administered dose that is not voided in the faeces is substantively retained in the GI tract wall rather than passing the intestinal barrier.

4. For the i.v. exposure scenario there was good correlation between the Ag tissue contents determined in the in vivo study and the PBTK model predictions for both AgNP and $AgNO_3$ (coefficient of determination: 0.92 and 0.89, respectively), with the model predicting comparable distribution (qualitatively and quantitatively) for both Ag forms.

(C) Relevance

1. The aim of this study was to obtain more precision on the mixture ratio kinetics of different Ag species – administered primary AgNP, Ag^+ formed from NP oxidative dissolution[52], and also secondary Ag nanoparticles[53] formed in vivo from dissolved ionic Ag – in order to probe their relative contribution to tissue distribution patterns. Most previous TK investigations have been unable to differentiate to what extent the various Ag species contribute to tissue distribution (as strong acid digestion techniques are a confounder). A combinatorial short-term TK study (i.v. and oral route) was coupled to a

[52] AgNP in aqueous solution (and physiological milieu), continuously release Ag ions by a process thought to involve cooperative oxidation, whereby AgNP react with dissolved oxygen and H^+ with the formation of peroxy intermediates prior to liberation of Ag^+ [Liu J and Hurt RH (2010) Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ. Sci. Technol. 44: 2169-2175].

[53] Previous work by other investigators, e.g. by van der Zande et al. (2012) has demonstrated putative formation of secondary AgNP in tissues after administration of ionic Ag forms (in this instance $AgNO_3$).

TK modelling[54] approach. Based on previous studies, it is predicted that transformation of ionic Ag to secondary NP occurs. However, in this report the authors put forward the novel proposition that most of the ionic silver when intravenously injected is systemically available as de novo-formed secondary nanoparticles.

2. The absence of conventional Ag in blood measurements precludes its use for the determination of basic TK parameters connected to systemic exposure via the oral route. It does provide some data on the relative absorption of ionic and AgNP forms, together with associated tissue distribution patterns. **Study rank for this purpose: SUPPORTING**. It should be noted that the data relates to male animals only.

3. Whereas some previous reports on tissue distribution following single-dose (Park et al., 2013) and repeat-dose administration (Loeschner et al. (2011; van der Zande et al., 2012) of sub-toxic amounts of Ag lead to the conclusion that ionic Ag forms have relatively greater systemic availability than AgNP, results from this study suggest that actually no difference exists. However, when a similar model had been applied previously to published TK data (in Bachler et al., 2013) it was concluded that ionic Ag had higher bioavailability than AgNP forms. The authors propose that size or coating differences in the various AgNP might explain the discrepancy in their in vivo results versus the other studies.

4. Taking the available robust TK studies into consideration, the majority do not support the notion that coating or size influences have a substantial impact on AgNP bioavailability, although one recent report using the i.v. route (Pang et al., 2016[55]) showed moderate effects attributable to some of the capping systems. This reviewer suggests that more attention instead be paid to the influence of ionic Ag in AgNP test materials (intrinsic or formed on storage) and whether this accounts for the variance in outcomes. Unfortunately, the Juling et al. work does not provide such specifics for their selected AgNP test article.

5. The publication does apply a novel PBTK approach applied to both elemental Ag and a soluble Ag form ($AgNO_3$). This, or a similar, model may be a valid approach to assessing TK parameters for various Ag reference substances, or at least to reducing the need for multiple conventional TK assessments. It is recommended that this possibility be further investigated.

6. The specific parameters reported in this study have only limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design (but note the recommendation in Point '5').

[54] An adaptation of a physiologically based toxicokinetic (PBTK) model specifically designed for ionic and NP Ag which is described in detail elsewhere [Bachler et al. (2013)]. Parameters applied in the PBTK model are described in an accompanying supplementary information paper.

[55] Pang C et al. (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.

(D) Study robustness and scientific reliability indicators

1. The selected AgNP (16 nm; citrate-capped) test article is previously characterised BAM reference material which is equivalent to an EC Joint Research Centre (JRC) nanoform known as NM-300. The AgNP characterisation procedures are only briefly described with the support of cross-referenced publications. They were evaluated as robust (taking into account the use of a previously well characterised reference material). Quantitation of silver in the AgNP stock suspensions that was present in the ionic form was stated as being performed. However, these results are not reported.

2. Concentration verification for dosing solutions was not reported.

3. The choice of phosphate-buffered saline as the vehicle for i.v. formulations is considered to be non-ideal (due to the potential for interaction of chloride ions with Ag^{+}) – an alternate such as isotonic glucose would have been preferable. It is not clear to what extent the choice of vehicle could have influenced outcomes of the i.v. study segment.

4. The group sizes were considered to be adequate for performance of a TK study.

5. Faecal samples were obtained via the use of individual metabolic cages in a manner which minimised the potential for contamination.

6. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not reported. Surfactant stabilising agents present in the nanoparticle formulations may influence TK factors such as distribution[56], and such surfactant coatings have been known to affect protein opsonisation of NP with secondary impacts on distribution and clearance. The investigators did not explore this possibility experimentally which was an oversight in view of the i.v. administration route. In this study, the surfactant stabilisers constituted 8% (w/w) of the test article, and therefore the calculated i.v. surfactant dose is not insignificant at 0.32 mg/kg bw. Surfactant negative control groups were a part of the experimental design.

7. The study was non-GLP.

[56] For example, see Tröster SD et al. (1990) Modification of the body distribution of poly(methyl methacrylate) nanoparticles in rats by coating with surfactants. Int. J. Pharm. 61: 85-100.

Liu et al., 2012 (In chemico / surrogate physiological media studies)

Test article: Silver nitrate ($AgNO_3$) Silver NP (~5 nm); citrate-capped

The principal AgNP (4-5 nm) was synthesised de novo by borohydride reduction from $AgClO_4$ in the presence of citrate. In some experiments commercially available uncoated AgNP (20–40 nm) and massive silver foil were used as reference silver materials.

Test systems and associated endpoints

Several physiological surrogate fluids and non-physiological in chemico systems were studied. These included:-

i. Gastric and pseudo-extracellular fluid surrogates – oxidative dissolution of Ag (these were simple surrogates without the addition of proteins and certain ionic and higher molecular weight organic components).
ii. Aqueous in chemico system – Ag-glutathione complex formation.
iii. Aqueous in chemico system – Ag-complex photodecomposition (in the presence of glutathione, cysteine (0.3 mM), or oxidized glutathione).
iv. Collagen gel system – Ag-complex photodecomposition.
v. Aqueous in chemico system – selenium reactions with Ag^0 and Ag_2S.

Principal analytical techniques

Various analytical approaches based on UV-vis spectrometry, plasmon resonance tracking, transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray diffraction (XRD), and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). The reader is referred to the paper for further details.

(A) Data as reported

1. For the AgNP-5nm test article, the effects of pH on oxidative dissolution in simple media were established. Estimates were also made of the effect of particle size on AgNP dissolution based on previously published kinetic reports. Refer to excerpted Figure:

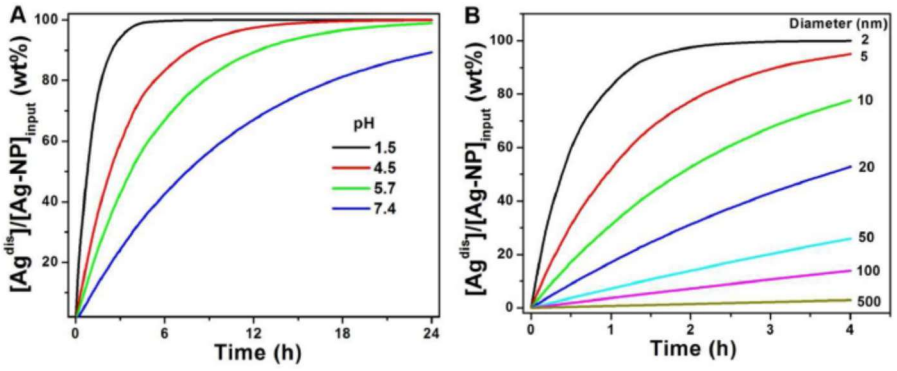


Figure 1. Effects of pH and particle size on Ag-NP oxidative dissolution in simple media estimated. (A) pH effect for 5 nm diameter Ag-NPs. (B) Primary particle size effect at pH 1.5 based on previously published reports [refer to publication for cross-references]. The calculations are reported as cumulative wt-% of total input silver released as ions.

It was further concluded that low pH in gastric fluid will lead to dissolution of AgNP in the stomach, but that dissolution will be incomplete for most NP due to limited residence time (10 – 240 min in stomach).

2. Silver was shown to undergo a strong binding reaction with the sulphur-containing biomolecule glutathione (GSH), viz.: $Ag^+ + GSH \leftrightarrow GS\text{-}Ag + H^+$. At high GSH/Ag ratios the stoichiometry of the complex was Ag-GSH, while at low ratios the stoichiometry tended to be Ag_2-GSH.

3. To explore chloride/thiol exchange reactions, AgCl precipitates formed by adding $AgNO_3$ to phosphate buffered saline were exposed to GSH, while tracking particle size by dynamic light scattering (DLS). Addition of GSH dissolved the formed micron-sized AgCl precipitates within 45 min to produce Ag-GSH complexes (monomers or soluble oligomers).

4. AgNP underwent rapid reactions between silver surfaces and reduced selenium species. Selenide was rapidly exchanged with sulphide in preformed Ag_2S solid phases.

(B) Data assessment and analysis

1. The dataset presented in excerpted Figure 1 may be useful in predicting AgNP dissolution characteristic in different tissue and cellular compartments, e.g. the stomach (pH 1.5); intra-lysosomal compartment (pH 4.5); and neutral pH situations such as extracellular fluid, blood, and interstitial lung fluid (pH 7.4).

2. Based on the overall findings from the study, the authors proposed that there is support for the hypothesis that AgNP are not absorbed to any extent after oral administration nor are they distributed within the body as intact particles (but rather in ionic form after dissolution). However, it is doubtful whether the simple modelling experiments reported in this work can substantiate such a definitive claim without supporting in vivo investigations. Whilst uptake of Ag^+ after AgNP dissolution is likely to be prominent in oral absorption, as discussed elsewhere in this review document it may not be the exclusive mechanism and uptake in particulate form may also occur; especially where small AgNP are involved.

3. Remarks on thiol reactivity and its relevance to absorption and transport/distribution are provided in the next section.

(C) Relevance

The study is relevant to consideration of the oral absorption phase for ionic and nanoparticulate Ag forms, and to complexities linked to both phase changes and chemical speciation fluxes in the G.I. tract; and also to transformations which may occur during distribution and in target tissues. **Study rank for this purpose: SUPPORTING**.

1. Only simple physiological fluid surrogates or aqueous reaction systems were used by the investigators, but useful information on the predicted chemical transformations of ionic Ag and AgNPs in biological media were obtained. These add to other published reports on the various forms of Ag complexation which occur with inorganic species and certain biomolecules ubiquitously found in vivo.

2. The potential for AgNP dissolution in low pH gastric fluid is in accord with several other reports (though it must be noted that only a protein-free system was utilised for this segment of the study – refer to Walczak et al., 2013 in respect of the relevance of protein components in gut milieu).

3. It is known that protein transport of Ag is significant in vivo (and this is discussed within the publication). It is proposed that due to the affinity of Ag^+ towards serum albumin and abundance of this protein in the blood (50 g/L in humans), it is likely to be an important high MW transporter of ionic Ag.

4. The study also confirms findings reported by other groups that Ag^+ binds avidly to glutathione, and therefore that this biomolecule will very likely have an important role during absorption across the gut cellular compartments, and thereafter during distribution/intra-corporeal transport processes. It is known that Ag-thiol complexes can exchange between different thiol groups despite the strong Ag-thiol bond. Hence it is reasonable to hypothesise that Ag-GSH complexes are likely to be important transporters for Ag in vivo (with subsequent thiolate ligand exchange being possible). Indeed, the authors predict that the majority of Ag in the systemic circulation will initially be bound to low MW thiol complexes with high binding affinities, but where Ag is still exchangeable (thereby giving Ag a significant biological mobility). Although sulphides and selenides have even higher binding affinities than these thiols, their concentration is much lower in physiological fluids, and hence they are unlikely to have a major role in distribution processes. Higher stability sulphide and selenide complexes appear to be more relevant to tissue fixed depots where this affinity leads to long-term sequestering of Ag.

5. Aspects of the work provide a basis for the chemical characterisation of argyrial Ag depots. In respect of argyria occurrence in vivo, the observation that reduced selenium species are able to react with Ag and AgS species appears important. The formation of AgNP (Ag^0 state) from photoreduction of Ag-thiol and Ag-protein complexes was a notable observation with relevance to skin argyria. The authors propose an integrated model for argyria whereby after Ag absorption, there is transport of Ag-thiol complexes which can then be transformed to stable thiol and selenide complexes in tissues (with additional photoreduction processes also operational in the case of the skin).

6. The investigators point out the pitfalls of quantitative Ag analysis in biological solutions where proteins are present which can bind Ag. For instance, tandem ultrafiltration-spectrometry approaches (e.g. for release rate measurement), are liable to exclude Ag^+ which is bound to protein and to then incorrectly assign this as being particulate in nature. Insoluble AgCl complexes are also subject to such experimental artefacts when ultrafiltration or ultracentrifugation is used. In situ plasmon resonance tracking methodology was suggested as an alternative.

7. In overview, the study can be viewed as complementary to conclusions arising from the study by Walczak et al., 2013 (which is reviewed later in this document). Ionic and nanoparticulate Ag behaviour in physiological conditions is subject to a number of complex and inter-related processes which impact on speciation and TK. A consequence is that the dynamic and complex nature of Ag transformations in biological media inhibits the straightforward application of conventional bioelution modelling for bioavailability estimation.

(D) Study robustness and scientific reliability indicators

1. The characterisation of the synthesised AgNP reference in respect of physical and chemical parameters was considered to be limited in extent.

2. The physiological surrogate fluids were only simple systems, e.g. lacking protein and some inorganic components. Therefore, some caution is required in not over-interpreting the findings reported in these models.

3. GLP status: Not applicable – in chemico behaviour and modelling studies.

Walczak et al., 2013 (G.I. tract surrogate media studies)

Test article: Silver nitrate ($AgNO_3$) Silver NP (60 nm); citrate-capped

Test systems

Saliva, gastric and small intestine fluid surrogates, with and without the addition of proteins. In the main experiments, the systems were integrated in a sequential manner.

System composition / experimental conditions

The compositions of the physiological surrogate systems were broadly in line with those previously used for bioelution/bioaccessibility investigations on various metals[57]. However, there were notable differences in certain respects, including the inclusion of various proteins in some of the experiments. Other notable divergences in experimental conditions included lower material loadings than typically applied in bioelution studies on metals.

Principal analytical endpoints

In the main surrogate physiological media experiments, particle dissolution, particle formation, and chemical speciation were evaluated using a battery of techniques:

a. Single particle ICP-MS (SP-ICPMS) – size and particle number concentration.
b. Dynamic light scattering (DLS) – hydrodynamic particle size.
c. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) – morphology and elemental composition of particles.

(A) TK data as reported in the publication

The reader is referred to the initial parts of the publication covering the initial characterisation of AgNP and $AgNO_3$ in various media and water. Results from the more important studies on AgNPs and Ag^+ ions ($AgNO_3$) in the in vitro digestive system model are summarised here.

1. G.I. tract surrogate media used to model the oral, gastric and intestinal compartments, were equivalent to those previously formulated by Versantvoort et al.[58]. The gastric medium was HCl-based. Refer to excerpted Table for compositional details:

Saliva (pH 6.8 ± 0.1)	Gastric juice (pH 1.3 ± 0.1)	Duodenal juice (pH 8.1 ± 0.1)	Bile juice (pH 8.2 ± 0.1)
896 mg KCl	2752 mg NaCl	7012 mg NaCl	5259 mg NaCl
200 mg KSCN	306 mg $NaH_2PO_4 \cdot H_2O$	3388 mg $NaHCO_3$	5785 mg $NaHCO_3$
1021 mg $NaH_2PO_4 \cdot H_2O$	824 mg KCl	80 mg KH_2PO_4	376 mg KCl
570 mg Na_2SO_4	302 mg $CaCl_2$	564 mg KCl	150 µl HCl (37%)
298 mg NaCl	306 mg NH_4Cl	50 mg $MgCl_2 \cdot 6H_2O$	250 mg urea
1694 mg $NaHCO_3$	6.5 ml 37% HCl	180 µl HCl (37%)	167.5 mg $CaCl_2$
200 mg urea	650 mg glucose	100 mg urea	1.8 g BSA*
290 mg amylase*	20 mg glucuronic acid	151 mg $CaCl_2$	30 g bile*
15 mg uric acid	85 mg urea	1 g BSA*	Milli-Q water
25 mg mucin*	330 mg glucosaminehydrochloride	9 g pancreatin*	
Milli-Q water	1 g BSA*	1.5 g lipase*	
	2.5 g pepsin*	Milli-Q water	
	3 g mucin*		
	Milli-Q water		
			Sodium carbonate solution
			84.7 g $NaHCO_3$
			Milli-Q water

*Protein components included in the "digestion with proteins" but not included in the "digestion without proteins".

Table I. Composition of the in vitro physiological surrogate fluids (content per 1000 ml fluid).
Reviewer remark: Note that both protein-free and protein containing variants were tested.

[57] For example: Eurometaux (2010) The Standard Operating Procedure for the Bio-Accessibility Testing Programme of Eurometaux, November 10; and Lombaert N et al. (2018) Use of bioelution as a screening tool for characterisation of substances. American Journal of Analytical Chemistry 9: 134-149.

[58] Versantvoort CH et al. (2005) Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem Toxicol 43:31–40.

2. SP-ICPMS analysis showed that during the whole simulated digestion process, with proteins present, AgNPs maintained a similar size (around 60 nm) and size distribution. However, particle number diminished significantly under the conditions present in the gastric fluid. Refer to the excerpted Figure:

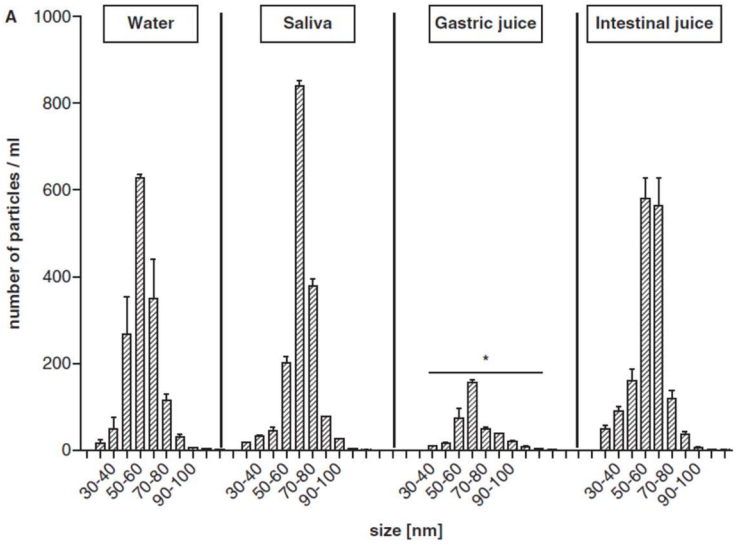


Figure 4. Size distributions of particles detected after digestion of 60 nm AgNPs measured with SP-ICPMS. The digestion was carried out in the presence of proteins normally present during human digestion. Sizes of AgNPs are shown in water, saliva, gastric and intestinal juice. On the left axis: numbers of particles per ml detected in the samples (average ± SEM). The total numbers of particles per ml in AgNP samples (A) were: 1460 ± 51 in water control, 1627 ± 22 in saliva, 374 ± 33 in the gastric juice and 1577 ± 72 in the intestinal juice. *Number of particles significantly different ($p < 0.05$) compared with saliva sample.

3. In the case of AgNP, the presence of proteins had a marked influence on the observed number of particles when comparing the gastric and intestinal fluids, with evidence of agglomeration occurring in the gastric surrogate medium. For the ionic silver form ($AgNO_3$), It is notable that the presence of protein in the gastric fluid had no apparent effect on the particle count (being undetectable), whereas a considerable formation of secondary AgNP (20 – 40 nm) was observed in the intestinal fluid when protein was included. Refer to the excerpted Table:

		Digestive stage (number of particles/ml and mean size of NPs as determined by SP-ICPMS)		
Type of material	Digestive conditions	Saliva	Gastric	Intestinal
AgNPs	Without proteins	1418 ± 137	125 ± 85*	149 ± 75*
		60 nm	20–30 nm#	20–30 nm#
	With proteins	1627 ± 22	374 ± 33*	1577 ± 72
		60 nm	60 nm and large clusters of 200–500 nm shown by SEM-EDX	60 nm
$AgNO_3$	Without proteins	ND	ND	ND
	With proteins	ND	ND	860 ± 811 20-40 nm

#The lower size detection limit of SP-ICPMS is 18–20 nm; *Number of particles significantly different ($p < 0.05$) compared with saliva sample; ND, number of nanoparticles/ml in the respective digestive juices was comparable with the number of nanoparticles/ml in water; AgNPs, silver nanoparticles; SEM-EDX, scanning electron microscopy with energy dispersive X-ray analysis; SP-ICPMS, single particle-inductively coupled plasma mass spectrometry.

Table II. Summary of the results of in vitro digestion of 60 nm AgNPs and $AgNO_3$.

4. In the gastric medium where AgNP showed evidence of clustering, SEM-EDX revealed that these clusters were composed of AgNPs and chlorine.

5. SEM-EDX examination of the secondary AgNP formed during intestinal medium incubation of $AgNO_3$ in the presence of proteins showed them to be composed of silver, sulphur and chlorine.

(B) TK data assessment and analysis

This investigation was not a conventional TK study, but instead examined aspects of solution behaviour of reference forms of an ionic Ag reference substance and a AgNP. Although the outcomes do inform on processes occurring under approximated physiological conditions relevant to oral absorption, the investigations were not standard bioelution assessments. Hence, they did not involve quantitative Ag dissolution values with linkage to bioavailability predictions. Therefore, it is not relevant to make the standard judgement on Confidence level for systemic exposure parameters.

1. Based on the results presented in Table II of the publication, it is inferred that in the absence of protein the dissolution of AgNP (and/or the formation of undetectably small NP) in the gastric medium was extensive (cf. particle number and size results for the saliva and gastric fluids). Due to the relationship between sphere mass and dimensions (which is proportional to the third power of the diameter), it should be noted that relatively small decrements in NP size can still represent a substantial degree of dissolution.

2. Whereas, with protein present in the gastric fluid, there is evidence of the formation of NP agglomerates (presumptively by chloride-bridging). This is in accord with previous reports, where it has been shown that similar AgNP undergo aggregation/agglomeration when exposed to low pH conditions[59]. When conditions are subsequently changed to model those of the small intestine, including a pH shift and change of chloride concentration, these agglomerates break down to re-form mainly 60 nm AgNP.

3. In respect of the results with $AgNO_3$ in the intestinal surrogate fluid containing protein, the investigators noted a very high degree of variability between the incubation sample replicates (triplicates) regarding the secondary NP formation (number and size distribution). This is notable given that the conditions were a simplified and tightly controlled in vitro situation, suggesting that AgNP formation from ionic Ag^+ in the presence of protein is a highly stochastic process which is complex to predict.

4. The formation of secondary NP from an ionic Ag reference substance ($AgNO_3$) in the intestinal fluid containing protein was shown to be substantial and is presumed to be due to protein corona formation which stabilises the small NP (including Ag-Cl-S linkages). However, the study design did not include experiments to directly evaluate how this might bear on Ag absorption from this G.I. tract compartment.

(C) Relevance

The study is relevant to consideration of the oral absorption phase for ionic and nanoparticulate Ag forms, and complexities linked to phase changes and chemical speciation changes in modelled physiological conditions of the G.I. tract. **Study rank for this purpose: SUPPORTING**.

1. The surrogate fluid compositions were more elaborate than those used in typical bioelution studies on metals, and are expected to be closer simulants to actual G.I. tract luminal conditions.

2. The main finding of the study is that under physiological conditions (in the presence of proteins), AgNPs probably reach the small intestine in their native form, whereas Ag^+

[59] El Badawy AM et al. (2010) Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. Environ. Sci. Technol. 44: 1260–1266.

ions undergo substantial post-gastric transformation to small secondary Ag-containing NPs. In the case of the latter, it is reasonable to assume that systemic absorption from the post-gastric G.I. tract includes NP uptake as well as Ag^+ solution-phase transfer (further complicated by equilibria between these two states). Furthermore, speciation work demonstrated that a series of Ag complexation effects also operate, some of which are labile forms whilst others are expected to be relatively stable and insoluble. The findings on secondary NP formation from Ag^+ are in accord with other publications [e.g. Liu et al., 2012; Juling et al., 2016)].

3. Therefore, as a high-level outcome, this study provides persuasive evidence that ingestion of either AgNPs or Ag^+ ions ultimately leads to intestinal exposure to AgNPs, albeit of differing characteristics.

4. The findings have important implications for bioavailability predictions for Ag based on simple bioelution models. It adds weight to the conclusion that Ag behaviour in physiological media is subject to complex and multi-factorial influences, and that due to this it will be problematic to attempt the prediction of real bioavailability using conventional bioelution modelling.

5. Notwithstanding its contribution to Ag speciation related to TK, the approach selected still represents a relatively simple investigative system. To focus on just one example, given it is an in chemico model it cannot account for a variety of important phenomena which operate at the interface of the luminal contents and the first cellular compartment. For instance, these include direct cellular uptake of particles, active Ag ion transport, and interactions with the very high levels of Ag-reactive glutathione known to be present in cells of the G.I. tract mucosa.

6. It is well-established that Ag is reactive toward sulphur-centres and it readily forms sulphur-complexes. This study confirms that such reactions are also important in the formation of the secondary NP from ionic Ag in the presence of protein in the small intestine (given that these NP include Ag-Cl-S linkages).

7. Unfortunately, the study was not of a design which would help predict the relative degree of absorption of either ionic Ag (or administered AgNP) in the stomach versus the intestine by the oral route.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation for physical parameters was considered to be robust, also taking account of the fact that a well-known commercial source of AgNP had been selected (sourced from NanoComposix, San Diego, USA). However, the investigators did not employ ultracentrifugation or other approaches in order to determine the level of ionic Ag present in the AgNP stock solutions. Experimental validation would have been improved if this parameter had been included, but given the endpoints of interest did not focus on quantitation of Ag in solution, it is considered that this omission did not have a major impact on the study reliability.

2. Physiological model validity/applicability is evaluated as appropriate in terms of it being a (simplified) simulation of mammalian G.I. tract lumen conditions. Equivalent simulants

have previously been applied to studies of other nanoparticle behaviour in the gut[60]. In this respect, it is notable that:

a. The sequential surrogate fluid transitions which were used represented a holistic "fed-model" rather than study of independent oral, gastric and intestinal situations as is commonly conducted with G.I. tract surrogate fluid studies.

b. The experimental conditions included certain protein components specific to the modelled G.I. tract compartment (refer to previously excerpted Table I). This is likely to be a closer proxy for real-life conditions than use of surrogate fluids which are protein-free.

3. The analytical approach taken was considered to be mainly reliable. It should be noted that the lower size detection limit of SP-ICPMS for AgNP is expected to be circa 20 nm and smaller particles than that would not be detected. Therefore, where the experimental findings demonstrated that particles were absent, it cannot be inferred with complete confidence that this due to Ag being present only in the dissolved state.

4. GLP status: Not applicable – modelling investigation.

[60] Peters R et al. (2012). Presence of nano-sized silica during in vitro digestion of foods containing silica as a food additive. ACS Nano 6(3): 2441–2451.

Loeschner et al., 2011 (4-week study)

Test article: Silver acetate (AgAc) Silver NP (14 nm); PVP-capped

Species: Rat Strain: Wistar

Single sex only (non-pregnant females)

Age: 5 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): Females ~110 g.

Oral (gavage) Repeated dosing: twice daily Duration: 28 days (4-wk)

AgAc: 9 mg/kg bw/d as Ag equiv.

Dose was not explicitly detailed (as AgAc nominal dose), but an equivalent Ag dose value is stated in the publication.

AgNP (14 nm): 9 mg/kg bw/d nominal; 12.6 mg/kg bw/d actual.

Note: ionic Ag content resulted in an ionic Ag dose of about 1 mg/kg bw/d.

Ag levels in plasma / urinary and faecal Ag levels / terminal Ag tissue levels

TK determinations: Terminal sampling only at d29 for plasma and Ag in tissues. Urine and faecal Ag levels determined during Week 3 (24h collection period).

Ag digestion/analysis methodology:

Nitric acid digestion / ICP-MS

(A) TK data as reported in the publication

1. Based on TK assessments conducted during Week 3, elimination of Ag via the renal route with urine was low (<0.1%), whereas a higher amount of silver was eliminated in the faeces, viz. 63 ± 23% of the daily dose for AgNP and 49 ± 21% the daily dose for AgAc. Refer to the excerpted Table:

	urine (µg)	feces (µg)	urine (% of 24 h intake)	feces (% of 24 h intake)
Ag-PVP nanoparticles	0.10 ± 0.05	1190 ± 430	0.005 ± 0.003	63 ± 23
Ag acetate	0.73 ± 0.23	610 ± 250	0.057 ± 0.017	49 ± 21

Absolute and relative amount of silver (mean ± 1s.d., N = 5) excreted in urine and feces within a 24 hour time period in week 3 of the study.

Table 1 Excretion of silver in urine and faeces (24 h)

2. When normalised to the achieved doses of 12.6 and 9 mg Ag/kg bw/day for the AgNP and the AgAc groups, respectively, the Ag concentrations in tissues from animals administered AgNP were consistently lower than those in animals treated with AgAc. For instance, approximately 40-50% less in the case of plasma, kidney, stomach and brain; and circa 20% less for liver in respect to AgAc comparator results. Refer to the excerpted Figure.

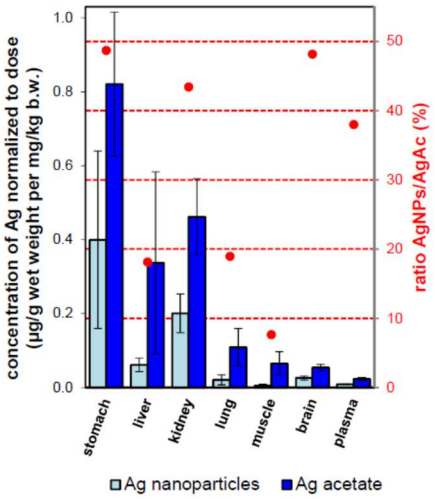


Figure SUPPLEMENTARY Silver concentration in rat organs (normalised in respect of administered Ag dose) following administration of either AgNP or AgAc. On the right-hand y-axis. the results for AgNP are ratioed in respect of the respective tissue concentrations achieved with AgAc.

(B) TK data assessment and analysis

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.

2. The high-level findings in relation to mass balance for urinary and faecal route elimination are consistent with several other studies on various Ag forms (administered via both the oral and i.v. routes), commencing with investigations by Furchner et al., 1968 and then replicated in further work (refer also to the separate commentary on Furchner et al., 1968).

3. As it is not possible to discriminate the extent of biliary excretion[61], this particular study cannot be used to derive an oral absorption value.

4. The distribution pattern of Ag following AgAc exposure resembled that reported in other studies where rats have been orally exposed to ionic Ag forms (such as AgCl or $AgNO_3$). Aside from depots associated with the gastrointestinal tract, Ag was found to be

[61] In order to determine biliary excretion, it would be necessary to design an oral route study involving cannulation of the bile ducts to estimate percentage of administered dose excreted via biliary route (and hence to directly calculate the extent of oral absorption). From i.v. studies in the rat using soluble Ag forms, it is known that in this species a substantial fraction of the administered Ag dose is eliminated via the biliary route (refer to Gregus and Klaassen, 1986; Klaassen, 1979); as further supported by Tichý P et al. (1986) Biliary excretion of 110mAg and its kinetics in the isolated perfused liver in rats. J Hyg Epidemiol Microbiol Immunol. 30: 145-148.

distributed to the liver, kidney, spleen, bone marrow, lymph nodes, skin, thyroid, heart, pancreas, adrenal glands, and brain.

5. The organ distribution pattern and concentrations of Ag following exposure to AgNP in this study were comparable to the results obtained from a 28-day oral repeat dose investigation (Kim et al. 2008) with 60 nm AgNP at about twice the dose level in this report (i.e. 30 mg/kg bw/d). A possible inference is that the bioavailability of the larger AgNP in the Kim et al. study was comparatively lower.

(C) Relevance

1. The study has only limited relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for elemental Ag and a soluble Ag form (AgAc).

2. The findings relating to elimination and to distribution are confirmatory since they appear congruent with several other TK studies on elemental and ionic Ag forms. It should be noted that the design included only female animals. The elimination mode dataset (see above section) and its quantitative assignments are also useful confirmation of some earlier reports.

3. The TK results have some relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. The ionic Ag compound used was the same, viz. AgAc. The TK study was performed in the same species (rat; young adult) though the design included only female animals. Route and dosing regimen are relevant (oral route; repeated dosing). However, the duration of dosing/terminal TK assessment was only 4 weeks. In terms of normalised Ag equivalent administered dose, the selected dose of AgAc (9 mg Ag/kg bw/d) sits between the mid- and high-dose selected in the Sprando et al./Babu et al. investigations[62]. The findings on the relatively low levels of Ag evident for the brain do have potential bearing on the EPMF EOGRTS design. **Study rank for this purpose: SUPPORTING.**

(D) Study robustness and scientific reliability indicators

1. Rather than being a reference source material, AgNP were synthesised de novo from $AgNO_3$ and then capped with PVP to prevent agglomeration. Based on the published details, the synthetic method was considered to be reliable.

2. AgNP characterisation procedures were evaluated as moderately robust. This included quantitation of silver in the AgNP stock suspensions that was present in the ionic form. It was determined that the AgNP test article contained quite high amounts of ionic Ag approximately 11% ionic form, corresponding to an ionic Ag dose of about 1 mg/kg bw/d.

3. Test article stability and homogeneity checks were performed. Concentration verification for dosing solutions was conducted. Assessments for storage-induced artefacts (such as serial measurements of ionic Ag release characteristic) were properly conducted.

4. The group sizes were considered to be adequate for performance of a TK study.

[62] Ag acetate administered at 0, 0.4, 4 or 40 mg AgAc/kg bw/day (equivalent to 0, 0.26, 2.6 or 26 mg ionic Ag/kg bw/day) to young adult Sprague–Dawley strain rats.

5. The use of a twice-daily (split) dosing regimen should be noted in any inter-study comparison with TK investigations based on a conventional single daily dose.

6. PVP was also added to the AgAc solution to a concentration of 11.5 mg/ml; it is considered unlikely that this minor amount of PVP complicates comparison of the results to other studies employing AgAc as a test article.

7. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

8. The study was non-GLP.

Lankveld et al., 2010 (5-day dosing period; associated with 17-day time-course experiment)

De Jong, 2012 (5-day dosing period; associated with 17-day time-course experiment)

Citation details for De Jong, 2012: Toxicokinetics and toxicity of nanosilver. Personal communication. The DeJong report related to the same TK experiments previously reported by Lankveld and co-workers, but provided some additional interpretations.

Test article: Silver NP (20 / 80 / 110 nm); coating type unstated

Species: Rat Strain: Wistar

Single sex only (males).

Age: 8 weeks old at the start of dosing.

Bodyweight (at commencement of dosing: unstated.
Note: animal bodyweight estimated at 300 g based on standard reference tables for this strain.

Intravenous (bolus; via tail vein) Repeated dosing: daily Duration: 5 days

IV: AgNP (20 / 80 / 110 nm) – estimated at 0.08 to 0.09 mg/kg bw/d.

Note: the publication does not state the exact administered dosages. This estimate is derived from the details provided in Table 1 of Lankveld et al. 2010, together with an inference based on bodyweight reference data for animals of this strain and age.

Ag levels in blood (inferred as whole blood)

TK determinations: d 1, 3 and 5.

Timepoints: 2, 5, 10, 20, 30 and 60 min.

Terminal Ag in whole blood / Ag tissue levels

24 h (d 2), 48 h (d 3), 96 h (d 5), 120 h (d 6), 168 h (d 8), 240 h (d 11) and 384 h (d 17). Comprising a relatively limited tissue set (liver, spleen, kidneys, lungs, heart, brain and testes).

Ag digestion/analysis methodology:

Nitric acid digestion at elevated temperature (microwave-assisted digestion was not utilised). Analysis was via ICP- MS.

(A) TK data as reported in the publication

1. Based on the dosing period time-course studies conducted during Days 1, 3 and 5, the blood Ag levels in all AgNP treated groups declined rapidly post-i.v. injection (with substantial clearance from the blood being evident by 10 min post-dosing). Refer to excerpted Figure:

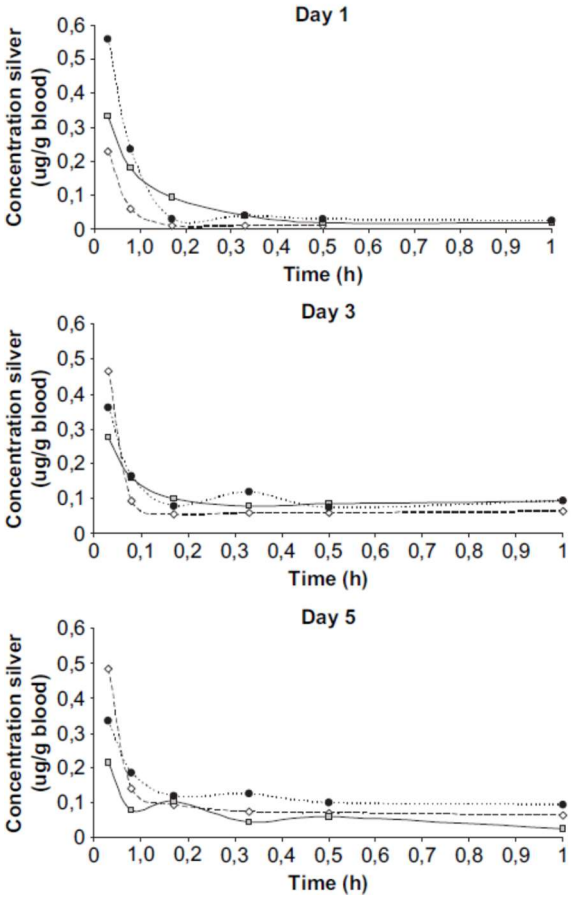


Figure 2. Blood concentration-time curves at day 1, 3 and 5. 20 nm: open diamond, 80 nm: grey square, 110 nm: closed circle (n = 2 for each time point). *Reviewer remark: There are errors in the x-axis label of the Day 1 and Day 5 graphs – the first timepoint should correctly be "0.1".*

2. Ag was detected in all the organs examined at terminal sacrifice. By the d5 timepoint, absolute Ag concentrations per gram of tissue were higher for the larger AgNP sizes, i.e. AgNP-110 nm ≅ AgNP-80 nm > AgNP-20 nm. Following both single and repeated i.v. administration, AgNP-20 nm distributed predominantly to the liver with lesser but approximately similar amounts being detected in the spleen and kidneys. For AgNP-80 nm and AgNP-110 nm, the pattern of distribution differed from the smallest AgNP (20 nm) wherein the rank order of observed distribution was spleen > liver > lungs > kidneys. Refer to excerpted Figure:

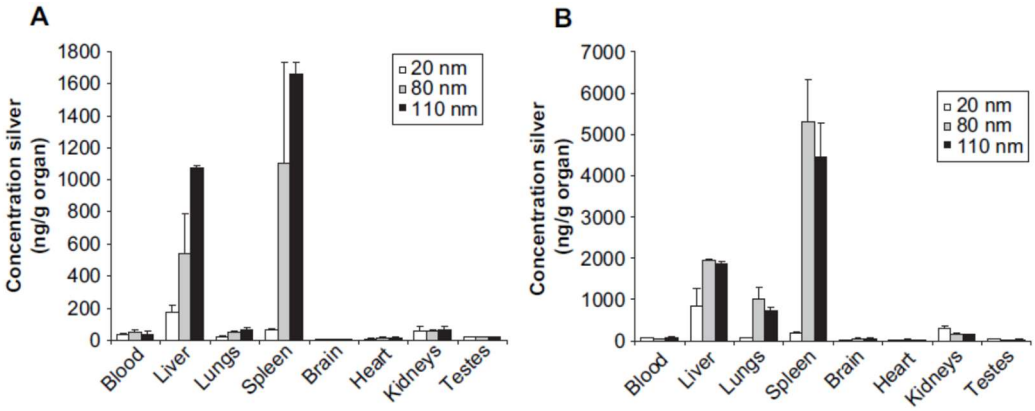


Figure 3. Mean silver concentrations (ng) per gram organ after one single injection (A) and 5 consecutive injections (B). 20 nm: white bars, 80 nm: grey bars, 110 nm: black bars; n = 3, error bars represent standard deviations.

3. In respect of solid tissues, the lowest Ag levels were evident in the case of brain, heart and kidney (irrespective of AgNP size). The investigators pointed out that for these tissues, the Ag levels were actually below those detected in the corresponding terminal blood measurements, and that the possible contribution of Ag in blood associated with the organs may need to be taken into account.

4. Time-course kinetics for Ag in tissues demonstrated that levels continued to increase after the first i.v. dose, typically still rising up to the point of the last dose (on d5). Refer to excerpted Figure:

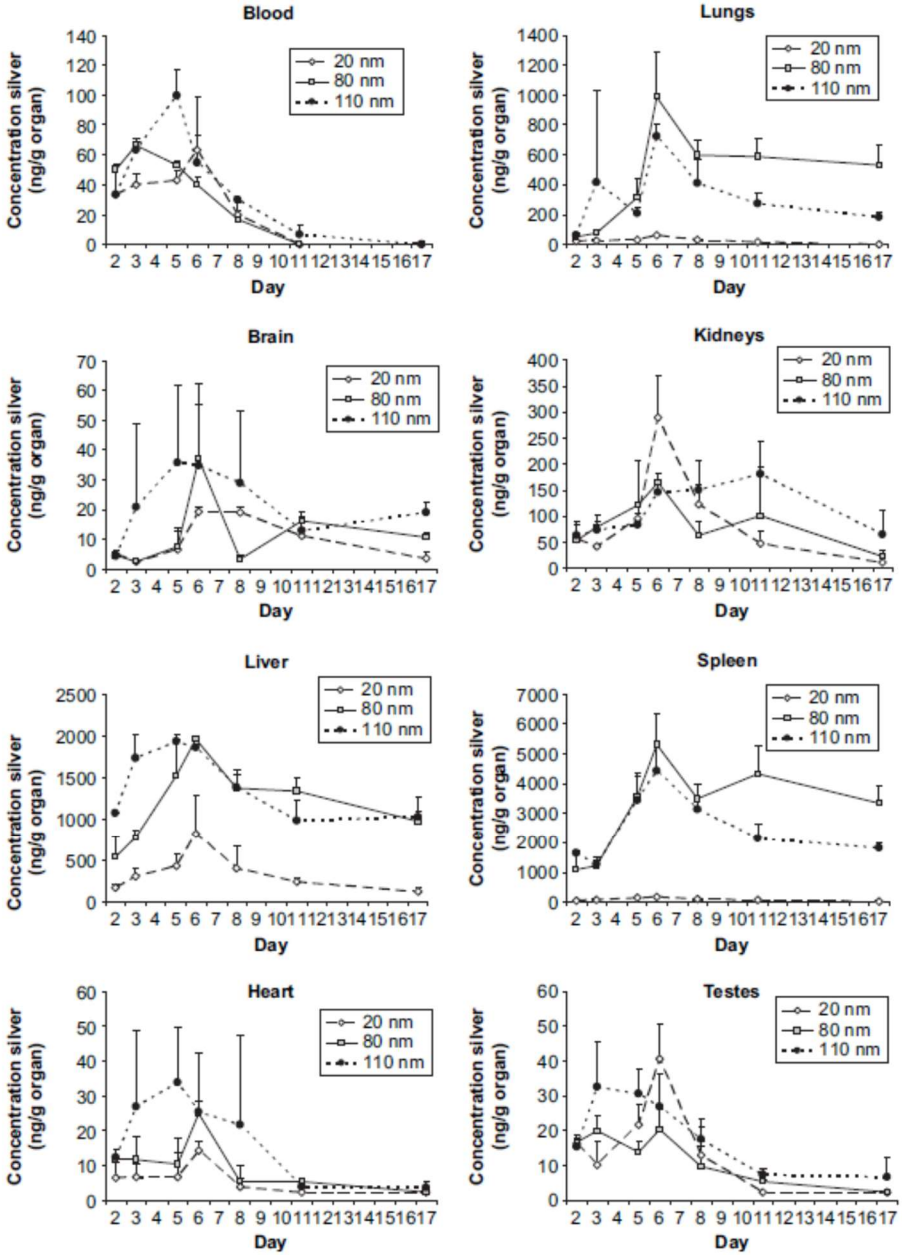


Figure 4. Mean silver concentrations (ng) per gram organ over time. 20 nm: open diamond, 80 nm: grey square, 110 nm: closed circle; n = 3, error bars represent standard deviations. *Reviewer remark: note the y-axis scale differences.*

5. Tissue clearance and persistence patterns are discussed in the next section.

6. A physiologically based toxicokinetic (PBTK) model was applied in order to model AgNP kinetics. There was reasonable agreement between measured data and model outputs. No clear correlation between AgNP size and TK parameters was noted.

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-RELEVANT for this publication due to the inherent study design. The absence of an oral route comparator treatment precludes any oral bioavailability estimation. In any inter-study comparison, it should be borne in mind that all values are derived from these investigations relate only to male animals.

1. Due to several considerations, including the low Ag dose levels used in this study and the restricted duration time-course for the serial blood Ag measurements, it was not considered to be of value to attempt to derive $AUC_{(0-t)}$ estimates.

2. Based on comparison of other i.v. route studies on AgNP where measurements are available following a single dose (e.g. Boudreau, 2012; and Park, 2011) it was evident that faster clearance from the blood occurred during this study (refer to excerpted Figure 2 [Day 1]). Whether this finding is due to the substantially lower total Ag dose used than in the other referenced investigations, or to other factors, is unclear. As discussed under Point D.1, some uncertainties concerning this published report affect inter-study comparisons.

3. In respect of Ag distribution patterns (concentrations per gram of tissue): as would be predicted, there was higher Ag uptake in organs which are part of the reticulo-endothelial system (liver and spleen). However, there was some AgNP size dependency in the differential levels observed – particularly when comparing spleen and liver levels for the smallest versus the two larger AgNP test articles. A firm hypothesis for the substantially lower Ag recovery in the case of AgNP-20 nm group was not put forward by the report authors. It could be partly due to greater renal elimination, which is known to occur in the case of smaller AgNP of circa 15 nm or less. The publication does not include sufficient particle size distribution data in order to gauge whether the AgNP-20 nm test article included a fraction of <20 nm. As investigation of elimination mode was not part of the experimental design, this remains a point of conjecture. It is also plausible that this particular AgNP underwent relatively greater dissolution to Ag^+ which was then eliminated more rapidly. It seems unlikely that the explanation could be a radically different distribution pattern to tissues other than those selected for measurement. In keeping with many other reports on a variety of small NP, it is important to note that total organ uptake of Ag by the liver was still significant for AgNP-20 nm (as indicated by Table 3A in the published report).

4. In this study, persistence of Ag depots after cessation of dosing was evident for the liver, spleen and lungs in the case of the larger size AgNP (80 and 110 nm). These Ag accumulations remained evident up to the point of the last measurement (at d 17).

5. Based on the tissue accumulation and elimination kinetics dataset (refer to excerpted Figure 4), it is clear that the absolute Ag concentrations associated with the brain and testis were low in the case of all the AgNP tested. Furthermore, there was evidence of elimination of Ag from these organs rather than a pattern of substantive persistence during the post-dosing period (up to d 17). For the testis, the d17 : d6 Ag tissue concentration ratios were 0.07, 0.15 and 0.22 for groups receiving AgNP-20 nm, AgNP-80 nm and AgNP-110 nm, respectively. In the case of the brain, the equivalent d17: d6 ratios were estimated as 0.21, 0.32 and 0.51 for AgNP-20 nm, AgNP-80 nm and AgNP-

110 nm, respectively. Whilst the brain retention value for the AgNP-110 nm group appears to be dissimilar and substantial, there was an apparent upward inflection of the Ag concentration between d11 and d17 for this treatment group which may have been spurious, leading to an artefactually higher value. In common with many other tissue distribution studies, the design did not examine to what extent the Ag depot was associated with the endothelial versus the intra-organ compartments.

(C) Relevance

1. Due to the i.v. route of administration, the study was not designed to quantitate systemic exposure via the oral route/ bioavailability.

2. Because of the i.v. route rather than use of oral administration, the study is informative in divorcing any potential influence on tissue distribution which might result from an oral absorption mode. **Study rank for this purpose: SUPPORTING.** Allied to its time-course design, this allows Ag tissue clearance kinetics (and the converse of persistence patterns) to be followed after a repeat dose regimen, i.e. repeated daily doses over a 5-day period, followed by successive measurements up to day 17 post-dose. However, it should be noted that based on the Ag in blood measurements, a steady state systemic exposure condition was only apparent for AgNP-20 nm, whilst this was not achieved for AgNP-110 nm, and a steady state condition was only approached in the instance of AgNP-80 nm.

3. Given the disparities in route of administration, duration of dosing, and dose levels, the TK results have limited relevance to direct interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. Furthermore, the study did not include an ionic Ag comparator substance.

4. Irrespective of considerations related to the chosen i.v. route of administration, the findings on Ag levels in the brain and testis add to the raft of conflicting data on Ag accumulation and retention patterns in these organs. In contrast to some other reports, only minor amounts of Ag were distributed to the testis and brain (irrespective of AgNP size), when compared to the other major organs of distribution. Also in contradiction to certain other studies, there was no evidence that clearance of Ag from these tissues was markedly retarded when compared to the other organs. Therefore, at the level of fundamental knowledge on Ag tissue distribution patterns related to the testis and brain, the results do have an indirect bearing on EPMF EOGRTS design in respect of TK considerations.

5. The PBTK model applied in this report was considered to be more simplistic than that described elsewhere (e.g. Bachler et al., 2013). For instance, in its consideration of the various compartments and fluxes, and in corrections for dissolved ionic Ag fraction.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures were evaluated as being moderately robust in respect of physical parameters, also taking account of the fact that a well-known commercial source of AgNP had been selected (sourced from NanoComposix, San Diego, USA). However, there were two notable omissions in the experimental approach: (i) the surface characteristics of the three sizes of AgNP were not specified; and (ii) the study apparently did not include quantitation of ionic silver concentrations in the AgNP stock suspensions. These important omissions complicate comparison of the findings of the study with other investigations on AgNP.

2. The publication makes no reference to test article stability and homogeneity checks (other than there being an absence of AgNP agglomeration). There was no mention of concentration verification for the dosing solutions. It was noted that the test article administered was apparently the AgNP dispersion as received from the supplier, and therefore the age of the dosing formulation is unknown (see also Point D.1.ii above).

3. The AgNP dispersion was in 2mM phosphate buffer, which is considered to be an appropriate i.v. dosing vehicle.

4. Silver analytical method quality control checks were performed including blood and tissue spiking and recovery assessments.

5. The group sizes were considered to be adequate for performance of a TK study (based on a serial terminal sacrifice).

6. Toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

7. The study was non-GLP.

Bergin et al., 2016 (Single-dose TK study)

Test article: Silver acetate (AgAc)
Silver NP (20 nm); PVP-capped / Citrate-capped
Silver NP (110 nm); PVP-capped / Citrate-capped

Species: Mouse **Strain: C57BL/6NCrl**

Single sex only (males)

Age: ~7 weeks old at the start of dosing.

Bodyweight (commencement of dosing): Unstated.

Oral (gavage) **Single dose**

AgAc: 10 mg/kg bw/d **6.5 mg/kg bw/d as Ag equiv.**

AgNP: 10 mg/kg bw/d.

Ag levels in faeces and urine / Terminal Ag tissue levels

TK determinations: Urine and faecal collection was performed at 0, 3, 6, 9, 12, 18, 24 and 48 h after the single oral dose ('cumulative collection at each timepoint' is inferred to be sub-group pooling of collected material). Terminal tissues samples were obtained for Ag quantitation at 48 h (comprising a limited tissue set). A terminal blood sample was not obtained.

Ag digestion/analysis methodology:

Nitric acid-microwave digestion / ICP-OES

(A) TK data as reported in the publication

1. Group time-course data for faecal Ag content are presented in Figure 2 of the publication. Peak elimination was evident at 9 h for AgAc, and between 6-9 h for the various AgNP; very little Ag was detectable 48 h post-dose.

2. Cumulative 0-48 h faecal Ag content values are provided in Table 2 of the publication. Refer to the excerpted Table:

			Ag in feces (% recovery of administered dose)	
Dosing group	Vehicle (coating)	Ag dose (mg/kg)	Mean	SD
AgNP 20 nm	2 mM citrate	10	98.6	32.5
AgNP 110 nm	2 mM citrate	10	84.0	16.6
AgNP 20 nm	10 kDa PVP	10	81.1	22.9
AgNP 110 nm	40 kDa PVP	10	70.5	18.4
AgOAc	Sterile H_20	10	84.0	17.0

Table 2. Cumulative Ag in faeces from 0 to 48 h after oral gavage with 10 mg/kg bw/d AgNP or AgOAc.

3. Urinary Ag measurements were below detection limits for nearly all samples; accordingly, no data were presented in the paper.

4. Terminal Ag tissue distribution data are presented in Table 3 (GI tract associated Ag) and Table 4 (liver, spleen and kidney) of the publication, respectively. Refer to the excerpted Table in respect of the latter results:

			Ag in liver (ng/g)[b]		Ag in liver (% recovery administered dose)		Ag in spleen (ng/g)[b]		Ag in spleen (% recovery administered dose)		Ag in kidney (ng/g)[b]		Ag in kidney (% recovery administered dose)	
Dosing group	**Vehicle (coating)**	**Ag dose (mg/kg)**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean[a]**	**SD**
AgNP 20 nm	2 mM citrate	10	16.87	11.43	0.008	0.005	22.94	9.78	0.0110	0.0050	2.62	0.81	0.0010	0.0003
AgNP 110 nm	2 mM citrate	10	11.19	7.45	0.005	0.003	24.45	11.13	0.0114	0.0053	2.12	0.20	0.0010	0.0001
AgNP 20 nm	10 kDa PVP	10	12.88	4.83	0.006	0.002	26.44	9.34	0.0128	0.0045	2.21	0.14	0.0011	0.0001
AgNP 110 nm	40 kDa PVP	10	8.11	6.65	0.004	0.003	33.17	18.11	0.0164	0.0091	2.43	0.20	0.0012	0.0001
AgOAc	sterile H_2O	10	301.32‡‡‡	162.50	0.144‡‡‡	0.810	94.44‡‡	60.37	0.045‡	0.029	12.54‡‡‡	6.77	0.0060‡‡‡	0.0034

[a] Significantly different from AgNP groups

‡‡‡ $p<0.0001$,

‡‡ $p = 0.002$,

‡ $p = 0.003$; one-way ANOVA, Tukey's multiple comparisons test.

[b] Note that mass recovery of Ag is expressed in ng/g tissue.

Table 4. Ag in tissues (liver, spleen and kidney) at 48 h after oral gavage with 10 mg/kg bw/d AgNP or AgOAc

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.

2. The high degree of faecal elimination observed (70% or greater) is quantitatively similar to estimates for AgNP and ionic Ag in other studies, such as Loeschner et al., 2011. Whilst the faecal Ag content findings for both the AgNP forms and AgAc are compatible with a pattern of predominantly biliary elimination, as reported elsewhere for rodent species, an actual quantitation of clearance via this mode is not possible from the study design (since it does not include biliary sampling). Neither is it possible to accurately derive an elimination half-life from the faecal Ag content time-course data.

3. No quantitative conclusions can be drawn on absorption characteristic from this study.

4. As would be expected based on the relatively low dose levels selected, the absolute Ag concentrations in the tissues were small in all treated groups. However, relatively higher Ag tissue content values (for liver, spleen and kidney) were evident following a single dose of AgAc (6.5 mg Ag/kg bw/d) in comparison to all groups receiving the 4 types of AgNP (10 mg Ag/kg bw/d) – particularly in respect of concentrations found in the liver[63] (refer to Table 4). This outcome is consistent with greater bioavailability in the case of AgAc.

5. In terms of reported tissue Ag levels for the studied AgNP, an obvious correlation with particle size was not apparent.

[63] Liver: less than 0.01% of the total silver for all AgNP groups versus 0.14% for AgAc.

6. Data confirming the ionic Ag content of the AgNP test articles was not included as part of the publication. If the ionic Ag content of the AgNP test articles was the same as previously reported by the group (Wang et al., 2014)[64], then a marked disparity between the various forms would be expected. In the Wang et al. work, the 110 nm AgNP (both coating types) was stated to have a low ionic Ag content (<1%), whereas the AgNP 20 nm (both coatings) contained higher ionic Ag concentrations (between 5 – 7%). It has previously been established in other investigations, e.g. van der Zande et al. (2012), that the ionic Ag content of AgNP formulations may influence TK outcomes, including apparent bioavailability. Unfortunately, the study did not include blood Ag level measurements which might have informed on this possibility, and organ Ag concentrations are not reliable for this purpose.

7. The qualitative pattern of Ag levels detected in the spleen, liver and kidney showed broad similarities to that observed in other studies with AgNP and ionic Ag forms, though with some variance in respect of inter-organ comparisons. However, these three organs were the only systemically exposed tissues investigated, and blood levels were not obtained as a further reference point, thus limiting the value of the study.

(C) Relevance

1. The study was not designed to quantitate systemic exposure via the oral route/ bioavailability.

2. The findings relating to elimination and to Ag distribution pattern are mainly congruent with several other TK studies in rodents examining elemental and ionic Ag forms. For investigations of elimination mode and associated kinetics, this type of study design is inferior to direct biliary elimination studies. In terms of any direct comparisons with other published work, it should be borne in mind that the test species was the mouse, and that the design included only male animals. **In view of this second species information on TK the study is categorised as: SUPPORTING.**

3. The results have limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. An ionic Ag compound was included (AgAc), at a dose level of relevance, via the oral route. However, the test species was the mouse (males only) rather than the rat, and the design included only a single oral dose. Other rodent TK studies–particularly in the rat–are considered to be more pertinent.

4. No reproductive tract tissues were examined in respect of their Ag levels.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures (partly cross-referenced to another report – see above) were evaluated as mainly robust for physical parameters. AgNP stock solutions were analysed for total Ag content. Quantitation of silver in the AgNP stock suspensions present in the ionic form is not specifically reported in the publication but this was previously evaluated in the cross-referenced report for the same test articles.

[64] Wang X et al. (2014) Use of coated silver nanoparticles to understand the relationship of particle dissolution and bioavailability to cell and lung toxicological potential. Small. 10: 385-398.

2. Test article stability checks were performed. Concentration verification for dosing solutions was not specifically reported. Assessments for storage-induced artefacts were not applicable due to daily preparation of dosing formulations.

3. The group sizes were considered to be adequate for performance of a TK study.

4. The digestion and analytical technique (ICP-OES) are not described in detail, but are cross-referenced to a publication which is known to represent a suitable methodology[65].

5. Urine and faecal samples were obtained with the use of metabolic cages in a manner which minimised the potential for contamination.

6. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

7. The study was non-GLP.

[65] Poitras EP et al. (2015) Development of an analytical method for assessment of silver nanoparticle content in biological matrices by inductively-coupled plasma mass spectrometry. Biol Trace Elem Res. 163: 184–192.

Furchner et al., 1968 (Single-dose study)

Test article: Silver nitrate ($^{110m}AgNO_3$); specific activity 8.7 Ci/g

Species: Multiple (refer to table below for further details)

Species	Strain	Sex	Age (mo.)	Body-weight	Dose (µCi) / Group size (single dose administered)		
					Oral	i.v.	i.p.
Rat	Sprague Dawley	Single sex only (males)	2.6–3.3	355 – 415 g	0.5 (n=6)	0.5 (n=6/n=6)*	0.4 / 0.5 (n=28/n=6)
Mouse	RF (inbred)	Single sex only (females)	2.5–3.1	~27 g	0.25 (n=12)	0.25 / 0.26 (n=12/n=12)*	0.25 / 0.35 (n=12/n=18)
Dog (Beagle)	-	Single sex only (males)	78 – 90	13.3 – 14.4 kg	0.6 (n=4)	0.6 (n=4)	Not performed
Non-human primate (Rhesus monkey)	-	Single sex only (males)	~48	6.7 – 6.9 kg	0.6 (n=4)	0.4 (n=4)	Not performed

*Two separate groups of the indicated number of animals received the test article either via the tail vein or jugular sinus.

Ag levels in faeces and urine

TK determinations: Urine and faecal collection was performed at periodic intervals (d1, d2, wk2, and wk3 post-dosing).

Terminal Ag tissue levels

Terminal tissue samples were obtained from rats or mice dosed via the i.p. route via serial sacrifices of groups of animals at intervals (d1, d4, d8, d12, d14, d18 and d22 post-dosing). The tissues evaluated by scintallography comprised the liver, spleen, kidney, heart, lung, intestine (with contents from oesophagus to rectum), skin/fur, testis, brain, and carcass. A terminal blood sample was obtained.

Ag digestion/analysis methodology:

Digestion was not required (scintillography was the detection technique).

Analysis was by 4~π liquid scintillation.

(A) TK data as reported in the publication

1. It was concluded that very little silver was absorbed from the gut via the oral route, with the cumulative elimination for all species being in excess of 90% of the administered dose by d2 post-dosing. Except in the dog, whole-body retention was observed to be <1 % of the initial dose within a week following administration (Figures 1-3).

2. Urinary **:** faecal elimination ratios are detailed in Table 4 of the publication. In all species and by all routes of administration more than 90% of the voided Ag dose was present in the faeces. Refer to the excerpted Table:

Species	1st day	2nd day	1st week	2nd week	Cumulative excretion at day 2(%)
Intravenous					
Mouse	0.030	0.017	0.028	0.095	82.08
Rat	0.010	0.007	0.009	0.031	70.73
Monkey	0.054	0.018	0.048	—	44.08
Dog	0.063	0.058	0.092	—	15.00
Intraperitoneal					
Mouse	0.012	0.053	0.019	0.059	88.46
Rat	0.006	0.009	0.013	0.044	77.00
Oral					
Mouse	0.001	—	—	—	99.61
Rat	0.001	—	—	—	98.35
Monkey	0.019	0.040	0.258	—	94.35
Dog	0.061	0.025	—	—	90.38

Table 4. Urinary: fecal excretion ratios for ^{110m}Ag

3. Individual tissue retention data for rats administered $^{110m}AgNO_3$ were presented in Figure 4 and 5. It was concluded that Ag retention in all tissues except the brain and spleen resembled the whole-body retention kinetics. Refer to the excerpted Figures:

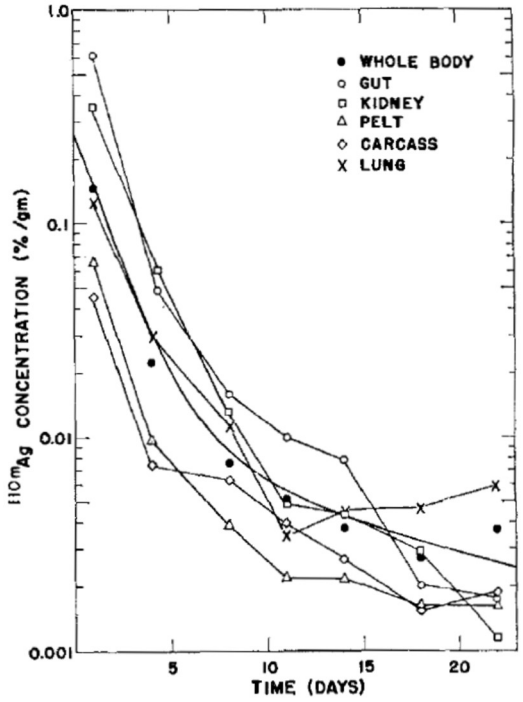


Figure 4. Concentration (% of injected dose/g) of ^{110m}Ag as a function of time. The smooth curve is a plot of effective retention after intraperitoneal injection in rats divided by the initial weight of the rats. The closed circles represent the average whole-body concentration of the 4 rats killed for each tissue data point. The other data points represent average concentration values for 4 rats.

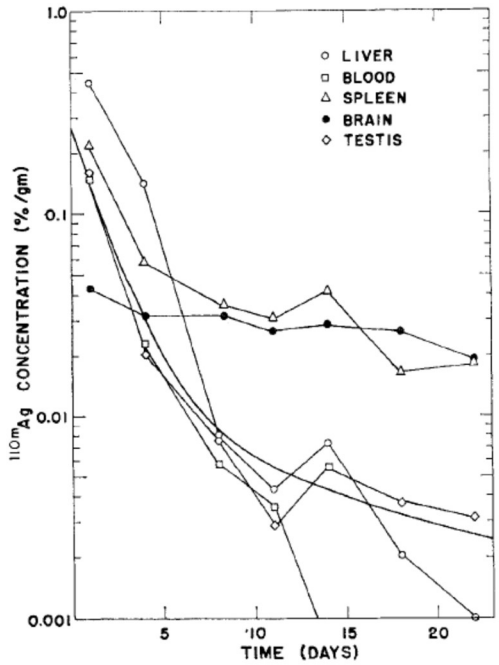


Figure 5 Tissue concentrations of ^{110m}Ag in rats. The concentrations that clearly deviate from whole-body retention are the spleen and brain <legend trunc..>.

(B) TK data assessment and analysis

Estimates only; subject to some imprecision. Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.

2. Ionic silver administered orally was quickly eliminated in both rodent species, and with comparable kinetics. The relatively greater amount retained by the dog after oral administration may be related to the longer GI tract transit time which is normal in this species versus rodents (circa 3x longer). Results in the non-human primate appeared to be intermediate between these norms.

3. Whilst the faecal Ag content data for $^{100m}AgNO_3$ are compatible with a pattern of predominantly biliary elimination, as has been reported subsequently for rodent species, an actual quantitation of elimination via this mode is not possible from the study design (since it does not include biliary sampling). Neither is it possible to directly calculate the extent of oral absorption in the absence of a specific biliary elimination study.

4. The tissue distribution dataset for ^{110m}Ag shows that reticulo-endothelial organs initially accumulate the highest levels (in terms of absolute specific activity values). Comparable distribution patterns have been replicated many times subsequently by other investigators. It is notable that the retention-elimination kinetics in all tissues, except brain and spleen, are qualitatively similar and mirror that seen for the whole-body analyses. This finding is consistent with the widespread argyric deposition pattern evident with Ag and Ag compounds seen in experimental animals and also for humans.

(C) Relevance

1. This investigation is significant in that it was the first systematic study to show that Ag elimination occurs mainly in the faeces and that only minor amounts are eliminated via the renal route. This was confirmed as a consistent pattern in 2 rodent (rat and mouse) and two non-rodent species (dog and monkey). It also confirmed that elimination kinetics are quite similar for the rat and the mouse (in the single sex studied).

2. The pattern of tissue distribution and the retention-elimination kinetics was established for multiple tissues, and has subsequently been studied by several other investigators (and is further reported elsewhere in this document). It is notable that the observed kinetics differed for the brain (and in this study also the spleen), with a more protracted tissue clearance half-time being evident.

3. Overall, useful data are included in respect of distribution and elimination modes in rodents (as well as other species). **Study rank for this purpose: SUPPORTING.**

4. The study has negligible relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for a soluble Ag form ($AgNO_3$).

5. Beyond definition of the fundamental modes of elimination relevant to ionic forms of Ag, the TK results have only limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design.

(D) Study robustness and scientific reliability indicators

1. The study was performed 50 years ago, prior to GLP, and the existence of best practice codification and guidelines for TK studies. Reporting of standard quality and control criteria is brief and lacking specifics. However, the originating institution was well-known as a centre of excellence with high academic credentials, and the investigators routinely performed distribution and elimination studies with metals/radionuclides. Therefore, it is considered likely that the experiments were diligently designed and performed to reasonable scientific standards.

2. The report does not confirm the use of individual metabolism cages for urine and faecal collection, nor procedures to prevent cross-contamination between these two excreta. It should be assumed that there was a potential for cross-contamination, though this is unlikely to have significantly affected the outcomes of the study.

3. The group sizes were considered to be adequate for performance of a TK study.

4. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

5. The study was non-GLP.

Pang et al. 2016

Test article: Silver NP of varying sizes; stabilised with a variety of capping agents:

Citrate – 36 nm

Polyethylene glycol (PEG) – 55 nm

Polyvinyl pyrolidone (PVP) – 36 nm

Branched polyethyleneimine (BPEI) – 63 nm

Silver nitrate ($AgNO_3$)

Species: Mouse **Strain: Balb/c**

Single sex only (males).

Age: 9 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): not specifically stated, but inferred as 20 g.

Intravenous (bolus; via tail vein) Single dose

AgNP – 1 mg/kg bw.

$AgNO_3$ – 1.56 mg/kg bw. / 1 mg/kg bw as Ag equiv.

Ag levels in blood (post-collection handling procedures were not detailed**)**

TK determinations: from d1.

Timepoints: 2, 10, 30, 60 min; and 6, 12, 24 and 72h post-dosing**.**

Terminal Ag tissue levels

Terminal tissues samples were obtained for Ag quantitation at 24 h post-dose.

Ag digestion/analysis methodology:

Nitric acid/peroxide-microwave digestion / ICP-MS.

(A) TK data as reported in the publication

1. Information on physical and analytical parameters for the various types of capped AgNP was presented in Table 1 of the publication. This included size, charge state (zeta potential data) and ionic Ag concentration. Refer to excerpted Table:

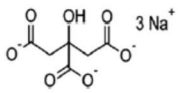
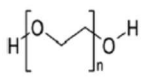
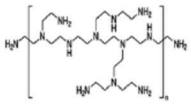
Name	TEM size (nm) (SD)	Hydrodynamic size (nm) Average (SD)	Zeta potential (mV)	Mass conc. (mg/ml)	Particle conc. (particles/ml)	Capping ligand (mg/ml)	Particles surface	pH	Solvent	Endotoxin (EU/ml)	Ag^+ conc. (µg/l)
Citrate AgNPs	28.7 (3.6)	35.8 (3.1)	−22.9	1.14	7.7E+12	0.017		7.1	Aqueous 2 nM Citrate	0.50	0.43 (0.10)
PEG AgNPs	32.9 (3.2)	54.6 (4.4)	−16.2	1.08	5.6E+12	0.160		6.5	Milli-Q water	0.69	0.45 (0.03)
PVP AgNPs	28.7 (2.1)	36.2 (3.0)	−22.1	1.07	7.7E+12	0.160		6.4	Milli-Q water	0.04	0.58 (0.10)
BPEI AgNPs	30.0 (3.0)	63.0 (3.6)	46.5	0.49	2.5E+12	0.066		8.1	Milli-Q water	0.36	0.80 (0.07)

Table 1. Physicochemical properties of Citrate AgNPs, PEG AgNPs, PVP AgNPs and BPEI AgNPs, as well as Ag+ concentration in stock suspensions.

2. Time-course data from the serial Ag in blood determinations were presented in publication Figure 6. Refer to excerpted Figure:

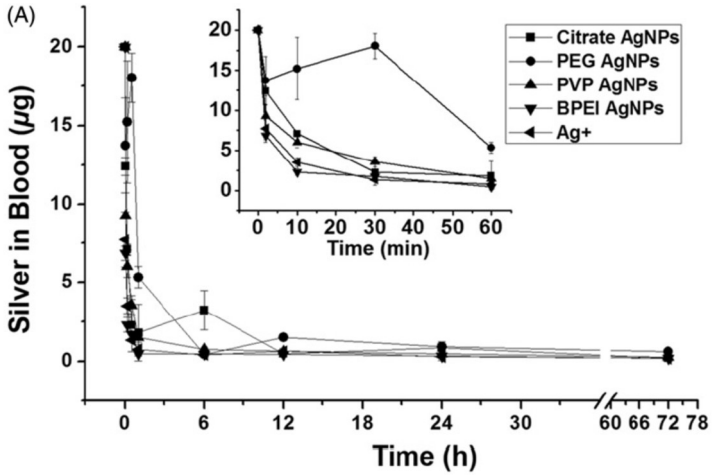


Figure 6A. Pharmacokinetics of AgNPs in mice. Citrate AgNPs, PEG AgNPs, PVP AgNPs, BPEI AgNPs and Ag^+ were injected into mice via a tail vein at a dose of 1 mg Ag/kg body weight per mouse (i.e. 20 µg Ag per mouse), respectively. Five mice were used at each time point. Data are expressed as means±standard deviation (n=5).

3. Derived TK parameters from the serial blood Ag measurements were calculated using WinNonLin version 5.2 (Pharsight, Princeton, NJ, USA) based on either non-compartmental model for PEG AgNP or else a two-compartment model for all other test articles (Table 2). Refer to excerpted the Table:

Parameters	Two compartment model				Non-compartment model
AgNPs	Citrate AgNPs	PVP AgNPs	BPEI AgNPs	Ag^+	PEG AgNPs
AUC (h.μg/g)	39.18	9.45	7.90	13.91	59.13
$t^{\alpha}_{1/2}$ (h)	0.11	0.02	0.05	0.10	51.65[a]
$t^{\beta}_{1/2}$ (h)	17.81	3.03	6.76	15.79	–
CL (g/h)	0.51	2.12	2.53	1.44	0.23
CLD2(g/h)	10.14	37.90	30.72	19.20	–
Vss (g)	12.66	8.86	23.24	30.87	14.18
V1(g)	2.03	1.02	3.10	3.14	–
V2 (g)	10.63	7.84	20.14	27.74	–

Table 2. Pharmacokinetics parameters of silver in blood after injection of AgNPs via tail vein for 72 h in mice (*sic*). AUC, area under curve; $t^{\alpha}_{1/2}$, first elimination half-life; $t^{\beta}_{1/2}$, terminal elimination half-life; CL, central compartment clearance; CLD2, peripheral compartment clearance; Vss, volume of distribution at steady-state in the central compartment; V1, volume of distribution in central compartment; V2, volume of distribution in peripheral compartment.
[a] $t_{1/2}$, elimination half-life.

The highest AUC values were evident for PEG AgNP (non-compartment model derivation) followed by citrate AgNP (two-compartment model). The findings for PEG AgNP correspond to the more retarded blood clearance for this AgNP type (see also remarks in the section 'TK data assessment and analysis').

4. Terminal tissue samples demonstrated that the highest Ag concentrations (publication Figure 5) were present in the spleen and liver irrespective of AgNP capping system. Ag concentrations in other organs were substantially lower, with the least Ag present in the brain. In terms of possible relationships with the nature of the capping system: mice treated with BPEI AgNPs had relatively greater lung Ag levels, whereas those treated with PEG AgNP had the highest spleen Ag levels (but see also remarks in 'TK data assessment and analysis'). Refer to the excerpted Figure:

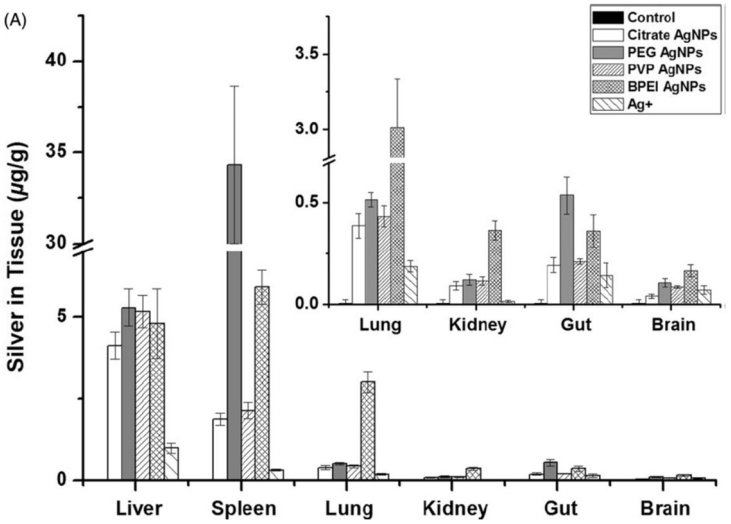


Figure 5. Biodistribution of AgNPs in different tissues. Mice were exposed to (controls), citrate AgNPs, PEG AgNPs, PVP AgNPs, BPEI AgNPs and Ag^+ by intravenously injection for 24 h (*sic*). Liver, spleen, lung, kidney, gut and brain were then harvested and concentration of Ag was determined using ICP-MS. (A) Tissue weight total silver amount per gram of wet-weight.

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-RELEVANT for this publication due to the inherent study design. The absence of an oral route comparator treatment precludes any oral bioavailability estimation.

1. BPEI AgNP had a markedly different charge state from the other NP types (zeta potential: +46.5 mV). The relatively greater uptake in the lung seen for this test article could be a result of its charge state, and it has been previously shown that NP cationic charge can lead to increased sequestering in the lung[66]. As a potential confounder, it should be noted that this particular AgNP also contained the highest amount of ionic Ag.

2. As well as surface capping agent (and related charge state) differences, there was some variation in size of the various AgNP used as test articles; from 36 – 63 nm based on average diameter measurements. Given the relatively narrow size range and the fact that small AgNP were not administered, it is considered that this variable is unlikely to have had a marked influence on the study outcomes. However, from the presented results it is not possible to make a firm conclusion as to whether the greater distribution to the spleen of BPEI and PEG AgNP was related to capping system or else their relatively greater size compared to the other AgNP (cf. Figure 5 and Table 1).

3. Based on the time-course results presented in Figure 6, Ag clearance from the blood after i.v. administration was rapid for both AgNP and Ag^+; being substantively complete by 30 minutes. The time-course curve for PEG AgNP differed with a comparatively slower clearance from the blood being evident. It is notable that PEG AgNP also showed less binding to blood serum albumin (BSA) in an ancillary in vitro binding assay. The lower protein opsonisation (protein corona formation) evident for PEG AgNP could lead to delayed reticulo-endothelial system scavenging, but as no tissue time-course measurements were conducted during the study, this remains a speculative possibility.

4. The Ag distribution in tissues corresponded qualitatively to the pattern seen in several other studies with AgNP with liver and spleen exhibiting the highest Ag concentrations.

5. Ag total body burdens for citrate, PVP and BPEI type AgNP were not dissimilar, but that for the ionic Ag reference substance was much lower (Table S1 of the publication). The higher total Ag body burden and percentage of retained dose values evident for the PEG AgNP was attributable to the greater uptake in the spleen. Refer to excerpted Table:

Organ	Citrate AgNPs	PEG AgNPs	PVP AgNPs	BPEI AgNPs	Ag^+
Liver	2.932(0.291)	3.765 (0.413)	3.672 (0.354)	3.410 (0.762)	0.696 (0.110)
Spleen	0.113 (0.011)	2.060 (0.258)	0.129 (0.015)	0.356 (0.031)	0.019 (0.001)
Lung	0.047 (0.007)	0.062 (0.004)	0.052 (0.006)	0.361 (0.039)	0.023 (0.004)
Kidenys	0.022 (0.005)	0.029 (0.006)	0.028 (0.005)	0.088 (0.011)	0.003 (0.001)
Gut	0.094 (0.018)	0.264 (0.045)	0.103 (0.007)	0.178 (0.039)	0.070 (0.030)
Brain	0.017 (0.004)	0.045 (0.009)	0.036 (0.002)	0.069 (0.012)	0.029 (0.008)
Total of Ag in organs (μg)	3.22	6.22	4.02	4.46	0.84
% of cumulative dose	16.12	31.12	20.10	22.30	4.20

Table S1. Mean silver concentrations (μg per organ, mean ± standard deviation), the total level of silver determined in all organs (μg), and the recovered percentage of the cumulative dose for AgNPs and Ag+ (20 μg per mouse, n=5).

[66] Fromen CA et al. (2016) Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells. Nanomedicine 12: 677-687.

6. It is unclear why Ag tissue concentrations were lower following treatment with the ionic Ag reference substance ($AgNO_3$) than for all AgNP types (Table S1 / Figure 5). This is an unexpected outcome.

(C) Relevance

1. Unlike more general investigations of AgNP TK, this study was specifically designed to probe the influence of several different capping systems (and linked parameters such as NP surface charge) on biokinetics. The fact that the dataset relates only to i.v. and not the oral route of administration is a consideration; it should be borne in mind that outcomes following parenteral exposure could differ from those via other routes of administration. Nevertheless, due to the systematic information on the relationship of capping system to TK outcomes, and also the paucity of other robust reports on this theme, the study is ranked as: **SUPPORTING**.

2. Overall, though some divergence in TK outcomes such as clearance and relative tissue distribution patterns were evident (e.g. for BPEI and PEG AgNP types), it appeared that the nature of the capping system had a relatively minor impact on the majority of TK parameters.

3. This TK investigation is considered to have negligible relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures in respect of physical parameters were considered to be robust. Surface chemistry and surface charge state were established as part of the primary experimental design. Quantitation of ionic silver concentrations in stock suspensions was measured but the experimental details provided are insufficient to allow judgement as to whether the methodology was fully valid, e.g. in discriminating true dissolved Ag concentration.

2. Concentration verification for dosing solutions was not reported as having been performed.

3. The use of a saline-based vehicle for i.v. injection is not optimal in the case of Ag test articles, though it is inferred that this vehicle was only used for the AgNP test articles whereas the vehicle for $AgNO_3$ was water.

4. Only limited information on the in vivo segment experimental procedures is provided in the publication, and several important details are unclear, e.g. animal bodyweight, and detail on the blood collection procedures (volume obtained from each animal, collection medium etc.).

5. From evaluation of the presented results, some interpretative errors were made by the authors in certain conclusions (e.g. in the Discussion section of the publication in relation to body burden outcomes). Furthermore, the opinion of this reviewer is that the magnitude of the TK differences attributed to the influence of capping systems was overstated by the authors. In terms of consistency with other reports and biological plausibility, the previous discussed outcomes with the ionic Ag test article are a concern.

6. The group sizes were considered to be adequate for performance of a TK study.

7. For Ag concentration determinations, the preparative microwave-based technique involved digestion with hydrogen peroxide/nitric acid mixture rather than nitric acid alone, which is a less commonly used technique. Data from internal standards and recovery values were not provided. Whilst there was no specific statement on analytical limit of quantitation, the expectation is that it would be a high sensitivity method.

8. The study was non-GLP.

Kim et al., 2008 (4-week study)

Test article: Silver NP (60 nm); uncoated

Species: Rat Strain: Sprague Dawley

Both sexes (non-pregnant females)

Age: 6 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): Males ~280 g; females 190g.

Oral (gavage) Repeated dosing: daily Duration: 28 days (4-wk)

AgNP (60 nm): 30; 300; 1000 mg/kg bw/d

Ag levels in <u>whole blood</u> / terminal Ag tissue levels

TK determinations: Terminal sampling only at d29 for blood and Ag in tissues (comprising a limited tissue set).

Ag digestion/analysis methodology:

Wet digestion (Nitric/Perchloric Acid) / AES

<u>(A) TK data as reported in the publication</u>

1. Terminal Ag tissue distribution data, including blood concentrations, are presented in Table 5 of the publication.

<u>(B) TK data assessment and analysis</u>

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data. See also the caveats described in the following sections on relevance and robustness assessment of this study.

1. The absence of data on serial blood Ag levels precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure.

2. In broad terms, the relative Ag distribution to the predominant sites of the GI tract (stomach), liver, kidney and lungs qualitatively corresponded to the pattern seen in other studies with elemental Ag (AgNP) and ionic Ag forms.

<u>(C) Relevance</u>

1. The study is of limited value for the following reasons: (i) some deficiencies in its design and robustness (refer also to the following section on study robustness); (ii) a restricted set of TK parameters; and (iii) a dataset based on only a single timepoint (terminal samples). The top two dose levels selected were significant and greater than in most other studies in the overall Ag TK dataset (300 and 1000 mg/kg bw/d., respectively).

2. It has negligible relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for elemental Ag. The terminal Ag tissue distribution data has some utility in comparative terms only. However, it should be noted that the concentration of any contaminating ionic Ag in the AgNP test article was undefined, which presents an interpretative impediment.

3. For the reasons given above – and also taking into account the high AgNP dose levels selected – this TK investigation is considered to have negligible relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design.

4. The study has been cross-referenced by other researchers in comparative evaluations of AgNP TK, and also used in pooled data work, e.g. for PBTK modelling. For those reasons it has not been designated as completely 'Excluded'.

(D) Study robustness and scientific reliability indicators

1. No information on AgNP characterisation procedures were provided in the publication, neither is there a cross-reference to other published work on characterisation. No OECD recommended NP associated physical and chemical parameters are cited. Quantitation of ionic silver concentrations in stock/dosing suspensions was not reported as being performed.

2. Test article stability and homogeneity checks were not described in the publication. Concentration verification for dosing solutions was not reported as performed. Assessments for storage-induced artefacts (such as serial measurements of ionic Ag release characteristic) were absent. Therefore, test article related verification and controls are categorised as non-robust.

3. With due regard for the issues described in '1' and '2', the overall AgNP characterisation is categorised as non-robust to the extent that it significantly impedes interpretation as a stand-alone study.

4. The group sizes were considered to be adequate for performance of a TK study.

5. The tissue digestion method diverged from the norms commonly associated with Ag tissue level analysis (perchloric acid was included). In the absence of supporting verification, such as recovery data and other controls, it is not possible to evaluate the impact of this methodological divergence.

6. The selected analytical method is expected to possess a relatively poor detection sensitivity.

7. Minimal toxicity was evident for the mid- and high-dose animals. Its extent was considered insufficient to interfere with TK outcomes.

8. The study was non-GLP.

Xue et al., 2012 (Single dose TK study)

Test article: Silver NP (15 nm); no definitive statement provided as to capping system.

Species: Mouse **Strain: ICR**

Both sexes (non-pregnant females)

Age: 5 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): Males ~25 g; females ~25 g.

Intravenous (bolus; via tail vein) Single dose

AgNP 120 mg/kg bw.

Ag levels in blood (post-collection handling procedures were not detailed**)**

TK determinations: d1.

Timepoints: 10, 20, 30 min and 1, 3, 6, 12, 24 h post-dosing**.**

Terminal Ag tissue levels

Two separate investigations:

6, 12 h and 1, 7, 14 d post-dose and;

1, 7, 14 d post-dose.

A limited tissue set was investigated (liver, spleen, kidneys, and lungs).

Ag digestion/analysis methodology:

Nitric acid/peroxide digestion at elevated temperature (microwave-assisted digestion was not utilised). Analysis was via ICP- MS.

(A) TK data as reported in the publication

1. The results of blood kinetic studies and derived TK parameters are presented in Figure 3 and Table 3 of the publication, including differential information for male and female animals. Refer to excerpted data:

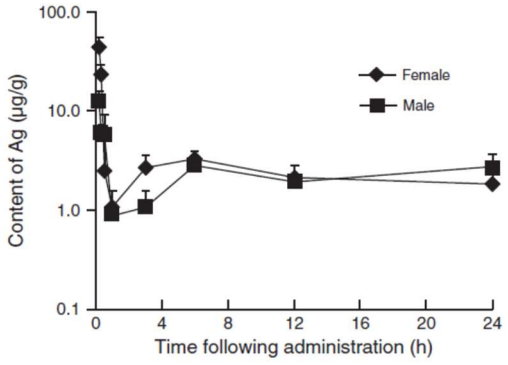


Figure 3. The blood concentration of silver as a function of time in mice.
Ten mice, five female and five male, were used at each time point.
Data are expressed as means ± standard deviation (n = 5).

Parameter	Female ($n = 5$)	Male ($n = 5$)
AUC ($\mu g\ ml^{-1}\ h^{-1}$)	163.9	127.0
$T_{1/2}$ (h)	29.9	15.6
MRT (h)	36.1	31.2
Cl ($ml\ h^{-1}\ g^{-1}$)	0.8	1.0
V_{dss} ($ml\ g^{-1}$)	31.9	21.7

Table 3. Kinetic parameters of silver nanoparticles in mice following a dose of 120 mg/kg via intravenous injection. The kinetic parameters were estimated by noncompartmental modeling analysis using Drug and Statistical Software (DAS 2.1.1). Data were determined based on the average levels at various experimental time points from individual female (n=5) or male (n = 5) mice, so no standard deviation can be shown for parameters. AUC, Area under blood levels extrapolated to infinity; $T_{1/2}$, elimination half-life; MRT, mean residence time; Cl, clearance; Vdss, volume of distribution under a steady-state condition of blood levels.

(B) TK data assessment and analysis

Confidence Level (systemic exposure parameters) = NON-RELEVANT for this publication due to the inherent study design. The absence of an oral route comparator treatment precludes any oral bioavailability estimation.

1. Some gender differences in respect of blood kinetics parameters were evident (refer to Table 3 / Figure 3).

2. In broad terms, the relative Ag distribution to the only tissues investigated (viz. liver, spleen, kidney and lungs) showed qualitative correspondence to the pattern seen in several other studies with AgNP. The rank order Ag distribution to spleen>liver>lung>kidneys was also congruent with a number of other publications on AgNP. The Ag levels determined in the spleen of female mice at d7 and d 14 was moderately higher than that for male animals (only minor differences were evident for the other tissues examined).

(C) Relevance

1. The study is of limited value due to several issues with its design and robustness (refer also to the section “Study robustness and scientific reliability indicators” for more details). This included the selection of an i.v. dose level which resulted in an excessively high bolus dose of AgNP being administered which introduces the uncertainty that first order kinetic processes could have been perturbed. Furthermore, as reported elsewhere[67], in the case of AgNP introduced via the i.v. route the surface nature/capping system of the NP can influence TK parameters – uncertainty as to the surface chemistry of the AgNP test article used in this study is a further significant impediment to its interpretation. Other TK studies via this route are considered to be more relevant and reliable.

2. It is noted that some information on gender-specific TK differences were established in the report which augment information published elsewhere.

[67] Pang et al., 2016.

3. This TK investigation is considered to have negligible relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design (also taking into account that the test species was the mouse rather than the rat).

4. Due to the issues identified in the following section, the study was ranked as **EXCLUDED**.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures in respect of physical parameters were limited. No information is presented on chemistry including whether a capping system was present or whether the test article was an uncapped nanoparticle. Although details for the supplier of the test article were provided, no specifics on product name were included.

2. Quantitation of ionic silver concentrations in stock/dosing suspensions was not conducted (though it was inferred that dosing suspensions were freshly prepared). Concentration verification for dosing solutions was not reported.

3. The use of a saline-based vehicle for i.v. injection is not optimal for Ag test articles.

4. The stated group sizes were considered to be adequate in the case of the serial blood measurements and the tissue distribution studies. However, there are discrepancies in respect of the number of animals described in the publication in the 'Materials and Methods' versus the 'Results' sections, and related uncertainties as to the actual number of animals sampled for each timepoint. Furthermore, the described 'averaging' procedure applied to TK parameter calculation (refer to Table 3 legend) is not considered to be robust.

5. Details of the methodology relating to post-collection processing of blood samples was omitted. Therefore, it is not possible to ascertain whether the reported Ag levels in blood were based on whole blood, serum or plasma. In addition, the blood volumes obtained from each animal were stated as being 0.8–1.0 ml; this volume is high compared to the estimated total blood volume and has the potential to cause artefactual effects.

6. For Ag concentration determinations, the digestion technique utilised a hydrogen peroxide/nitric acid mixture rather than nitric acid alone, which is a less commonly used technique. Data from internal standards and recovery values were not provided. However, the reported LOD via ICP-MS analysis showed high sensitivity (0.001 ppb).

7. The AgNP dose level selected (120 mg Ag/kg bw.) was stated to cause toxicity based on results obtained in a separate experiment (e.g. histopathological changes in the liver and lung). In terms of a single i.v. bolus, it represents a high dose level and considerable dose rate with a potential to shift biokinetic processes from first to zero order. For these reasons the study is an outlier relative to most other TK investigations on Ag substances utilising this route of administration. Part of the rationale mentioned by the authors for selection of such a high dose level was for ease of Ag detection in tissues, but this appears to be a non sequitur given that a high sensitivity analytical approach was applied. Overall, it is not possible to make a definitive judgement on whether interference with TK outcomes may have occurred, but this possibility cannot be discounted.

8. The study was non-GLP.

Lee et al., 2013 (4-week study; with extended 4-month recovery period)

Test article: Silver NP (10 nm / 25 nm); citrate-capped

Species: Rat **Strain: Sprague Dawley**

Both sexes (non-pregnant females).

Age: 5 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): males 190 g; females 170 g.

Oral (gavage) **Repeated dosing: daily** **Duration: 28 days**

Recovery period: 120 days (with staged sacrifices after 30, 60 and 120 days of recovery)

AgNP (10 nm / 25 nm) – 0; 100; 500 mg Ag/kg bw/d.

Ag levels in <u>whole blood</u> (EDTA anticoagulant) / Terminal Ag tissue levels

Blood samples (heparinised) and tissues for Ag concentration determination were obtained at the end of the treatment period (d29) and at the staged sacrifice points during the recovery period (i.e. after 30, 60 and 120 days recovery).

Ag digestion/analysis methodology:

Nitric acid-microwave digestion / AAS

<u>(A) TK data as reported in the publication</u>

1. Terminal blood Ag concentrations are reported in Figure 3 of the publication. Ag was stated to be rapidly cleared after cessation of treatment. Refer to the excerpted Figure:

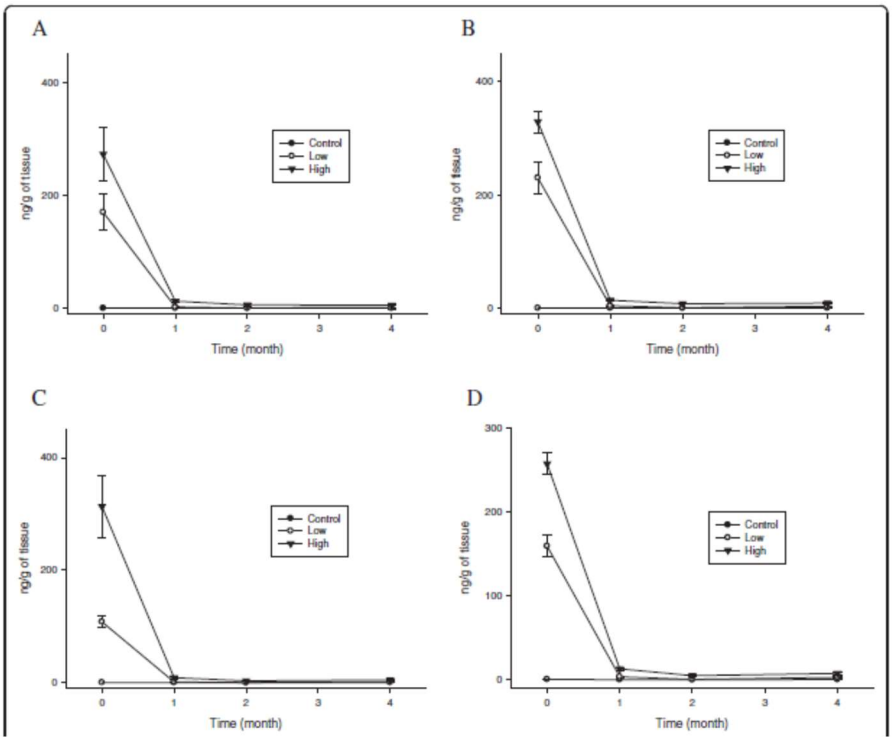


Figure 3. Blood silver concentrations (ng/g wet weight) before and after cessation of silver nanoparticle administration. The rats were allowed to recover for 1, 2, and 4 months following 28 days of oral silver nanoparticle exposure. A] Male (AgNP-10 nm). B] Female (AgNP-10 nm). C] Male (AgNP-25 nm). D] Female (AgNP-25 nm).

2. For AgNP-10nm (500 mg Ag/kg bw/d), the elimination half-life ($t_{1/2}$) for Ag in blood was calculated[68] to be circa 99 or 78 days for male and female rats, respectively. Longer elimination half-life ($t_{1/2}$) values were derived for the equivalent high dose group which received AgNP-25 nm, viz. circa 133 and 140 days for males and females, respectively. The datasets are detailed in Table 1 of the publication.

3. Ag elimination half-life data for various solid tissues following cessation of dosing are presented in Table 1 (liver, spleen, kidneys, brain, ovaries or testes), whereas the absolute Ag content time-course datasets for each organ are depicted in Figures 4-8 of the publication (refer to next section for further commentary).

(B) TK data assessment and analysis

Estimates only; subject to imprecision. Confidence Level (systemic exposure parameters) = LOW (raw data unavailable; design involving serial sacrifices with significant intervals between sample points is non-optimal).

1. The absence of data on serial blood Ag levels during the dosing period (e.g. during week 1 and week 4) precludes the derivation of $AUC_{(0\text{-}t)}$ and estimates of total systemic exposure by a conventional TK approach.

2. In general, the AgNP size (either 10 nm or 25 nm) appeared to have limited influence on the reported TK parameters.

3. The derived elimination half-life ($t_{1/2}$) values for Ag in blood presented in this study (refer to previous section) are considered to be problematic. Since raw data for the various groups are unavailable in the publication it was not possible to independently verify the TK model calculations. Irrespective of that impediment, a study design involving a staged sacrifice with the recovery phase blood sampling first conducted only after 4 weeks post-dosing will inevitably introduce imprecision in respect of $t_{1/2}$ estimates. The precision inferred in the estimates made by the study authors is misleading.

4. Similar design limitations afflict the other organ/tissue samples after cessation of dosing, though the clearance from solid tissues is generally thought to be a slower kinetic process, and is in part associated with lower mobility Ag complexes (selenide and sulphur complexes). Therefore, it might be thought that it would be possible to draw inferences from the relative elimination half-life values for inter-group and inter-NP type comparisons, as well as between various tissues. However, marked inconsistencies in $t_{1/2}$ values for the same organ are evident, e.g. for the liver when values are compared across the treatment groups and sexes for AgNP-10 nm. The absence of an obvious rationale for these differences affects the confidence level which can be attributed to the study.

5. External reviewers of the paper could conclude that the brain clearance $t_{1/2}$ values were consistently longer for the low-dose AgNP groups when compared to the respective high-dose groups (both sexes). It is obscure why such an inverse dose-related effect should be the case rather than, for example, evidence of saturation kinetics for the high-dose.

[68] Calculated from terminal Ag in blood concentration versus the time profiles using a nonlinear least squares regression model; WinNonlin (Pharsight, Cary, NC, USA).

6. Based on absolute Ag values in the various tissues (q.v. publication Figures 4-8), and if taken at face value, a more rapid clearance pattern is evident for the liver, spleen, kidneys and ovaries compared to the brain and testis. The authors propose that the persistence effect is due to the existence of a blood tissue barrier in the case of the slower clearance tissues (i.e. a blood brain barrier or blood testis barrier). However, they do not offer a hypothesis as to why the Ag concentrations in the testis continue to rise after cessation of dosing for both AgNP high-dose groups (up to the final recovery sacrifice at 4 months post-dosing).

(C) Relevance

1. The study has only limited relevance to the determination of basic TK parameters connected to systemic exposure via the oral route for elemental Ag.

2. The findings relating to clearance processes and to distribution of Ag are confirmatory since they appear largely qualitatively congruent with several other TK studies on elemental and ionic Ag forms. However, for reasons that have been previously stated, the actual kinetics values are not considered to be reliable and the study was ranked as **EXCLUDED**.

3. The TK results have limited relevance to the interpretation of the Sprando et al./Babu et al. studies and to the EPMF EOGRTS design. The TK study was performed in the same species (rat; young adult; both sexes used). Route and dosing regime are relevant (oral route; repeated dosing). However, extrapolation of findings of this study is limited by several considerations: (i) an ionic Ag reference substance was not included; (ii) the duration of dosing was only 4 weeks prior to further recovery period investigations related to Ag clearance processes; (iii) there were some non-optimal aspects of study design in respect of clearance parameters; (iv) the AgNP dose levels were greater than most other TK studies on Ag, and the total Ag dose administered was correspondingly high.

4. One positive aspect of the study as a reference point in the overall AgNP TK dataset is that the investigators did properly assess the stock AgNP formulations for ionic Ag levels, and that the Ag^+ contaminant concentrations were determined to be negligible. The use of freshly prepared dosing formulations also means that the influence of storage-induced Ag^+ accumulation can be disregarded.

5. If reservations about the design and internal discordance of the tissue clearance data are set aside then it would be concluded that clearance kinetics are more protracted in the case of tissues with a penetration barrier (brain and testis), and that therefore there are implications in respect of persistence and/or accumulation. As separately covered in this review document (including in the 'Conclusions' section) the various rodent TK studies show clear disparities in respect of whether Ag persistence in the brain and testis represents a real effect.

(D) Study robustness and scientific reliability indicators

1. AgNP characterisation procedures were evaluated as mainly robust for physical parameters (also taking into account a cross-referenced paper with further details). Quantitation of silver in the AgNP stock suspensions that was present in the ionic form was performed – only negligible Ag+ concentrations were reported at 0.0008% and 0.002% for the 10 nm and 25 nm AgNP, respectively.

2. Test article stability and homogeneity checks were not specifically described, nor was concentration verification for dosing solutions. Consideration of potential storage-induced artefacts is not relevant as the dosing solutions were freshly prepared each day.

3. The group sizes were considered to be adequate for performance of a TK study.

4. AAS as a detection technique for Ag may lead to relatively poor limit of detection, but the methodology in this study achieved acceptable sensitivity.[69]

5. Marked toxicity was not apparent in treated rats. In particular, significant hepatotoxicity was not observed even at the high dose (based on histopathology findings). Certain liver-related serum biochemical parameters showed limited increases in groups which received AgNP versus the control values after 28 days of treatment (with subsequent recovery being evident). Most other biochemical and haematological parameters were unaffected by treatment. Nevertheless, it should be noted that the AgNP dose levels employed (100 or 500 mg Ag/kg bw/d) in this study were high, particularly in respect of the high dose group. The possibility that toxic effects could have subtly influenced TK outcomes cannot be completely excluded, and such high doses of AgNP in the gut may cause absorption processes to shift to zero order.

6. In terms of scientific reliability, some findings with low biological plausibility were reported (refer to '(B) TK data assessment and analysis').

7. The study was non-GLP.

[69] The limit of detection was reported as 0.24 ppb and the limit of quantification as ~0.8 ppb.

Qin et al., 2016 (4-week study)

Test article: Silver nitrate ($AgNO_3$) Silver NP (>25 - <50 nm)[70]; PVP-capped

Species: Rat Strain: Sprague Dawley

Both sexes (non-pregnant females).

Age: 4 weeks old at the start of dosing.

Bodyweight (at commencement of dosing): Males ~73 g; Females ~68 g.
Note: Refer to remark D.1.

Oral (gavage) Repeated dosing: daily Duration: 28 days (4-wk)

$AgNO_3$: 0.5; 1 mg/kg bw/d ~0.32; 0.64 mg/kg bw/d as Ag equiv.

AgNP: 0.5; 1 mg/kg bw/d.

Ag levels in <u>plasma</u> / terminal Ag tissue levels

TK determinations: Terminal Ag in blood sample (d28) and Ag in a limited set of tissues (liver, kidney, testis, spleen).

Ag digestion/analysis methodology:

Nitric acid-microwave digestion / AAS

<u>(A) TK data as reported in the publication</u>

Not presented for the reasons detailed in the following sections.

<u>(B) TK data assessment and analysis</u>

Confidence Level (systemic exposure parameters) = NON-DEFINABLE for this publication due to the absence of key raw data.

<u>(C) Relevance</u>

Given the limitations and anomalies detailed in the section on study robustness, and the availability of superior information elsewhere covering Ag tissue and blood in rats following repeated oral dosing, it was concluded that the study should be **EXCLUDED** from further consideration in respect of the overall Ag TK dataset.

[70] Of variable characteristics due to synthesis by a biosynthetic reduction without further size selection.

(D) Study robustness and scientific reliability indicators

1. The animals were described as SD strain rats of 4 weeks of age at commencement of dosing. The stated bodyweights in the publication (see also Table I) are very low for this strain and age of rat. This issue raises concerns over the provenance of the animals, and how the results can be age and animal-status matched to other TK studies.

2. The AgNP studied was synthesised de novo from $AgNO_3$ via a bio-reduction technique, using a fungus (*Gibberella* sp). As reported in the publication, this resulted in AgNP with a wide particle size distribution, which complicates comparison with other studies using more homogenous AgNP test articles. Overall, the AgNP characterisation procedures were evaluated as sub-optimal for a complex AgNP which had been synthesised de novo.

3. No details are provided of test article stability and homogeneity checks, nor concentration verification for dosing solutions. It is unclear whether stock solutions were monitored for ionic Ag content if stored. It is unstated as to whether dosing solutions were freshly prepared, or not.

4. The tissue set taken for Ag determinations was limited (4 solid organs); and it did not include reproductive tract tissue from females (only the testis from male animals was evaluated for Ag levels).

5. The group sizes were considered to be adequate for performance of a TK study.

6. Overt toxicity of a nature considered sufficient to interfere with TK outcomes was not apparent.

7. The study was non-GLP.

APPENDIX 1

Ag in tissue distribution data from Boudreau et al. 2016: Table S3.

Summary of Ag concentrations in organs and tissues of Sprague Dawley rats in the 13-Week study of AgNP and AgOAc

Female Rats

AgNP - 10 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	**30 ± 12***	194 ± 33†	391 ± 156†‡♀	436 ± 83†‡♀
Blood, Wk-12 (ng/ml)	**23 ± 6***	177 ± 16†‡♀	192 ± 12†‡♀	244 ± 13†♀
Bone marrow, femur (ng/ml)	**3 ± 0***	15 ± 7†	17 ± 4†‡♀	9 ± 2†
Heart (ng/g)	**2 ± 0***	722 ± 103†‡♀	2136 ± 468†‡♀	5582 ± 597†‡♀
Ileum (ng/g)	**126 ± 99***	4199 ± 1154†	20037 ± 7849†♀	22332 ± 5112†
Jejunum (ng/g)	**9 ± 2***	7569 ± 952†‡♀	25556 ± 3561†‡♀	46590 ± 5665†‡♀
Kidney (ng/g)	**7 ± 2***	54810 ± 10372†‡♀	114544 ± 11003†‡♀	205730 ± 54023†‡♀
Liver, lateral (ng/g)	**6 ± 3***	4923 ± 505†‡♀	11892 ± 1790†‡♀	19333 ± 1856†‡♀
Lymph node, mes. (ng/g)	**9 ± 3***	149130 ± 25427†♀	212389 ± 33236†♀	148710 ± 27821†
Colon, proximal (ng/g)	**10 ± 2***	43640 ± 13890†♀	81778 ± 16055†♀	124070 ± 19625†
Spleen (ng/g)	**16 ± 8***	12653 ± 1396†‡♀	24278 ± 3249†‡♀	52360 ± 8008†‡♀
Uterus (ng/g)	**89 ± 49***	850 ± 143†‡♀	1658 ± 218†♀	3398 ± 327†‡♀

APPENDIX 1 (cont.)

AgNP - 75 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	**30 ± 12***	114 ± 13	154 ± 16†	196 ± 14†
Blood, Wk-12 (ng/ml)	**23 ± 6***	107 ± 11†	136 ± 12†	191 ± 16†
Bone marrow, femur (ng/ml)	**3 ± 0**	9 ± 3	5 ± 1	12 ± 2†
Heart (ng/g)	**2 ± 0***	151 ± 51†	560 ± 178†	1400 ± 529†
Ileum (ng/g)	**126 ± 99***	1709 ± 670	7113 ± 3002†	32144 ± 18954†
Jejunum (ng/g)	**9 ± 2***	2682 ± 673†	8502 ± 2733†	21259 ± 4915†
Kidney (ng/g)	**7 ± 2***	13966 ± 2769†	29105 ± 9157†	62140 ± 16377†
Liver, lateral (ng/g)	**6 ± 3***	1218 ± 456†	4021 ± 1367†♯	6046 ± 1700†
Lymph node, mes. (ng/g)	**9 ± 3***	113010 ± 11709†	144060 ± 18112†	126380 ± 18332†
Colon, proximal (ng/g)	**10 ± 2***	15124 ± 2456†	46726 ± 11693†	53960 ± 6117†
Spleen (ng/g)	**16 ± 8***	3415 ± 755†	8588 ± 1582†♯	15105 ± 3467†
Uterus (ng/g)	**89 ± 49***	287 ± 67	5983 ± 5261†	1010 ± 223†

AgNP - 110 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	**30 ± 12***	128 ± 27†	106 ± 10	198 ± 33†
Blood, Wk-12 (ng/ml)	**23 ± 6***	104 ± 13†	129 ± 15†	143 ± 12†
Bone marrow, femur (ng/ml)	**3 ± 0**	4 ± 1	5 ± 1	7 ± 2
Heart (ng/g)	**2 ± 0***	52 ± 10	615 ± 233†	1274 ± 462†
Ileum (ng/g)	**126 ± 99***	3286 ± 1432†	5231 ± 1889†	9710 ± 2672†
Jejunum (ng/g)	**9 ± 2***	2011 ± 945†	4126 ± 666†	13787 ± 2274†
Kidney (ng/g)	**7 ± 2***	9170 ± 1648†	24735 ± 9295†	52700 ± 6313†
Liver, lateral (ng/g)	**6 ± 3***	343 ± 63	1116 ± 290†	4349 ± 959†
Lymph node, mes. (ng/g)	**9 ± 3***	68367 ± 10711†	86140 ± 9686†	141060 ± 23876†
Colon, proximal (ng/g)	**10 ± 2***	9696 ± 1976	27047 ± 6416†	78461 ± 16909†
Spleen (ng/g)	**16 ± 8***	2056 ± 426†	4209 ± 685†	9427 ± 1339†
Uterus (ng/g)	**89 ± 49***	139 ± 31	8172 ± 7881†	728 ± 144†

APPENDIX 1 (cont.)

AgOAc[Ø]	Water/MC	100 mg/kg	200 mg/kg	400 mg/kg
Blood, Wk-1 (ng/ml)	53 ± 28*	1171 ± 91†	1631 ± 188†	9047 ± 1883†
Blood, Wk-12 (ng/ml)	18 ± 4*	792 ± 95†	1753 ± 212†	6960 ±
Bone marrow, femur (ng/ml)	3 ± 0*	800 ± 168†	3973 ± 1314†	13433 ± 2385†
Heart (ng/g)	76 ± 69*	375244 ± 310684†	116150 ± 18153†	189000 ± 5686†
Ileum (ng/g)	33 ± 21*	259000 ± 53550†	262000 ± 34697†	588667 ± 61167†
Jejunum (ng/g)	12 ± 5*	336333 ± 52145†	574900 ± 49119†	1416667 ± 133708†
Kidney (ng/g)	14 ± 9*	1481444 ± 205799†	1895000 ± 202640†	2526667 ± 188886†
Liver, lateral (ng/g)	4 ± 1*	759556 ± 69202†	1377600 ± 159674†	843667 ± 132640†
Lymph node, mes. (ng/g)	10 ± 3*	830444 ± 107536†	2119000 ± 237821†	5556667 ± 788973†
Colon (ng/g)	9 ± 2*	793778 ± 61053†	1175800 ± 87121†	1178000 ± 232975†
Spleen (ng/g)	24 ± 19*	560111 ± 85092†	2373000 ± 247557†	2843333 ± 101708†
Uterus (ng/g)	67 ± 37*	56844 ± 6010†	76350 ± 5098†	126100 ± 40771†

Ø Dose levels are expressed as mg/kg bw/d silver acetate.

The limit of quantification (LOQ) for endpoint of interest is as follows: blood (20 ng/ml), bone marrow (5 ng/ml), heart (4 ng/g), ileum and jejunum (13 ng/g), kidney (10 ng/g), liver (5 ng/g), mesenteric lymph node and colon (13 ng/g), spleen (10 ng/g), and uterus (22 ng/g).

* In the control groups, signifies significant linear dose trend effects (P≤0.05) based on Bonferoni adjustments.

† Signifies values that are significantly different (P≤0.05) from the control group by Bonferoni adjustments.

‡ In the 10 nm AgNP dose groups, signifies values that are significantly different from the 75 nm AgNP group at the same dose level.

♀ In the 10 nm AgNP dose groups, signifies values that are significantly different from the 110 nm AgNP group at the same dose level.

♯ In the 75 nm AgNP groups, signifies values that are significantly different from the 110 nm AgNP group at the same dose level.

Male Rats

AgNP - 10 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	37 ± 18*	131 ± 14†	238 ± 47†‡♀	346 ± 129†‡
Blood, Wk-12 (ng/ml)	18 ± 8*	166 ± 40†‡♀	174 ± 14†‡♀	218 ± 17†♀
Bone marrow, femur (ng/ml)	4 ± 2*	9 ± 3	22 ± 9	24 ± 7†
Heart (ng/g)	3 ± 1*	239 ± 95†‡♀	765 ± 191†‡♀	1424 ± 245†‡♀
Ileum (ng/g)	91 ± 53*	2112 ± 511†	36249 ± 27532†	45196 ± 18368†
Jejunum (ng/g)	107 ± 97*	2445 ± 448†	8695 ± 1363†‡♀	16262 ± 1434†‡
Kidney (ng/g)	7 ± 2*	3595 ± 441†‡♀	8372 ± 1905†‡♀	11159 ± 1258†‡♀
Liver, lateral (ng/g)	17 ± 13*	900 ± 260†‡♀	2984 ± 929†‡♀	3838 ± 646†‡♀
Lymph node, mes. (ng/g)	14 ± 4*	80660 ± 13718†	153320 ± 18382†‡♀	103370 ± 9808†
Colon (ng/g)	27 ± 18*	23350 ± 14940†	13759 ± 5528†	65301 ± 21581†
Spleen (ng/g)	5 ± 0*	5139 ± 1197†‡♀	15505 ± 2629†‡♀	26440 ± 2658†‡♀

AgNP - 75 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	37 ± 18*	106 ± 14†	105 ± 10†	135 ± 14†
Blood, Wk-12 (ng/ml)	18 ± 8*	87 ± 8	120 ± 10†	141 ± 11†
Bone marrow, femur (ng/ml)	4 ± 2	7 ± 2	8 ± 2	10 ± 3
Heart (ng/g)	3 ± 1*	40 ± 10†	83 ± 29†	191 ± 65†
Ileum (ng/g)	91 ± 53*	8797 ± 4714†	10190 ± 7438†	20378 ± 8347†
Jejunum (ng/g)	107 ± 97*	949 ± 196†	3277 ± 1240†	5642 ± 1483†
Kidney (ng/g)	7 ± 2*	1398 ± 209†	2348 ± 446†	5970 ± 2582†
Liver, lateral (ng/g)	17 ± 13*	215 ± 83†	237 ± 45†	1319 ± 694†
Lymph node, mes. (ng/g)	14 ± 4*	51548 ± 13798†	75320 ± 9207†	96770 ± 13368†
Colon (ng/g)	27 ± 18*	14607 ± 5920†	25227 ± 17521†	17046 ± 8598†
Spleen (ng/g)	5 ± 0*	1322 ± 391†	2488 ± 510†	6053 ± 962†

APPENDIX 1 (cont.)

AgNP - 110 nm	CIT/CMC	9 mg/kg	18 mg/kg	36 mg/kg
Blood, Wk-1 (ng/ml)	37 ± 18*	88 ± 7	102 ± 15†	557 ± 405†
Blood, Wk-12 (ng/ml)	18 ± 8*	109 ± 27†	98 ± 9†	108 ± 7†
Bone marrow, femur (ng/ml)	4 ± 2	5 ± 1	8 ± 3	6 ± 1
Heart (ng/g)	3 ± 1*	45 ± 20†	124 ± 77†	267 ± 93†
Ileum (ng/g)	91 ± 53*	1732 ± 671	7625 ± 2896†	38870 ± 26237†
Jejunum (ng/g)	107 ± 97*	1068 ± 284	2232 ± 901†	6683 ± 1388†
Kidney (ng/g)	7 ± 2*	1232 ± 216†	1315 ± 120†	3036 ± 352†
Liver, lateral (ng/g)	17 ± 13*	185 ± 62†	157 ± 44†	525 ± 156†
Lymph node, mes. (ng/g)	14 ± 4*	49072 ± 12157†	35050 ± 7376	97100 ± 12277†
Colon (ng/g)	27 ± 18*	4093 ± 1591†	2560 ± 805†	29171 ± 15125†
Spleen (ng/g)	5 ± 0*	1398 ± 583†	1425 ± 410†	3570 ± 682†

AgOAc[Ø]	Water/MC	100 mg/kg	200 mg/kg	400 mg/kg
Blood, Wk-1 (ng/ml)	27 ± 8*	1121 ± 151†	2482 ± 546†	12270 ± 1846†
Blood, Wk-12 (ng/ml)	34 ± 15*	595 ± 45†	1618 ± 227†	
Bone marrow, femur (ng/ml)	3 ± 0*	1020 ± 430†	6302 ± 1189†	
Heart (ng/g)	2 ± 0*	58500 ± 5696†	77330 ± 8890†	
Ileum (ng/g)	19 ± 10*	228920 ± 28619†	357100 ± 46308†	
Jejunum (ng/g)	12 ± 5*	423800 ± 43898†	929100 ± 189193†	
Kidney (ng/g)	8 ± 3*	440000 ± 60950†	768000 ± 96142†	
Liver, lateral (ng/g)	3 ± 0*	472620 ± 66058†	813300 ± 115528†	
Lymph node, mes. (ng/g)	7 ± 0*	549200 ± 45321†	1661400 ± 215854†	
Colon (ng/g)	18 ± 7*	310100 ± 44888†	658100 ± 52337†	
Spleen (ng/g)	6 ± 1*	601000 ± 138471†	1207700 ± 214244†	

Group prematurely terminated

Ø Dose levels are expressed as mg/kg bw/d silver acetate.

The limit of quantification (LOQ) for endpoint of interest is as follows: blood (20 ng/ml), bone marrow (5 ng/ml), heart (4 ng/g), ileum and jejunum (13 ng/g), kidney (10 ng/g), liver (5 ng/g), mesenteric lymph node and colon (13 ng/g), spleen (10 ng/g), and uterus (22 ng/g).

* In the control groups, signifies significant linear dose trend effects (P≤0.05) based on Bonferoni adjustments.

† Signifies values that are significantly different (P≤0.05) from the control group by Bonferoni adjustments.

‡ In the 10 nm AgNP dose groups, signifies values that are significantly different from the 75 nm AgNP group at the same dose level.

♀ In the 10 nm AgNP dose groups, signifies values that are significantly different from the 110 nm AgNP group at the same dose level.

♯ In the 75 nm AgNP groups, signifies values that are significantly different from the 110 nm AgNP group at the same dose level.

APPENDIX 2

Studies relevant to transplacental or embryo-fetal TK

KEY TD = Ag tissue distribution data nd = Not determined nc = Insufficient data to calculate

Dose level: □ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw

Authors	Title of publication	Ag form	Route	Species / Test system	Dosing regimen	Dose-level	Maternal TK	Trans-placental Ag transfer [Note 1]	Embryo-fetal TK
Philbrook et al., 2011	The effect of TiO_2 and Ag nanoparticles on reproduction and development of Drosphila melanogaster and CD-1 mice, Toxcol. Appl. Pharmacol. 257: 429–436.	Ag particles (220 nm)	Oral	Mouse (♀)	Single dose only GD 9	□ to □□□	nd	Not applicable	Qualitative only; tissue localisation.
Austin et al., 2012	Distribution of silver nanoparticles in pregnant mice and developing embryos. Nanotoxicology 6: 912-922.	AgNP (50 nm)	i.v.	Mouse (♀)	GD 7, 8, 9	□	Multiple tissues / Placenta	~0.01%	Whole embryofetal Ag level.
Lee et al., 2012	A transfer of silver nanoparticles from pregnant rat to offspring. Toxicol Res. 28: 139–141. [Short Communication]	AgNP (8 nm)	Oral	Rat (♂+♀)	TG 422 ≡	□□□ (Single dose level)	nd	nc	TD: Liver, kidney, lung, **brain**. nd: **lymphoid tissues, bone marrow**.
Melnik et al., 2013	Transfer of silver nanoparticles through the placenta and breast milk during in vivo experiments on rats. Acta Nat. 5: 107–115.	^{110m}AgNP (35 nm)	Oral	Rat (♀)	GD20 / LD14-16	□	Multiple tissues / Placenta	0.09 – 0.15%	Whole embryofetal Ag level. Lactational study TD: GI tract, liver, kidneys, spleen. nd: **brain, lymphoid tissues, bone marrow.**
Wang et al., 2013	Evaluation of the biological fate and the transport through biological barriers of nanosilver in mice. Curr Pharmaceutical Design 19: 6691-6697.	$AgNO_3$ AgNP (25 nm)	i.p.	Mice (♂+♀)	4-week premating	□	Placenta. [Note 2]	<0.01 – 0.13%	Whole embryofetal Ag level.
Wu et al., 2015	Effects of prenatal exposure to silver nanoparticles on spatial cognition and hippocampal neurodevelopment in rats. Environ Res. 138: 67-73.	$AgNO_3$ AgNP (20--50 nm) [Note 3]	i.v.	Rat (♀)	GD 10-18 (alternate days)	□□	nd	nc	Quantitation restricted to brain hippocampal Ag levels.

Note 1: Based on total Ag mass concentrations and expressed as the percentage of the Ag dose administered to dams.

Note 2: Other tissues were assessed via parallel measurements in non-pregnant adult mice.

Note 3: Two types of AgNP (uncapped and capped variants).

APPENDIX 2 (continued)

Studies relevant to transplacental or embryo-fetal TK

KEY TD = Ag tissue distribution data nd = Not determined nc = Insufficient data to calculate

Dose level: □ <=10 □□ >10 <100 □□□ >100 mg Ag equiv./kg bw

Authors	Title of publication	Ag form	Route	Species / Test system	Dosing regimen	Dose-level	Maternal TK	Trans-placental Ag transfer [Note 1]	Embryo-fetal TK
Austin et al., 2016	Distribution and accumulation of 10 nm silver nanoparticles in maternal tissues and visceral yolk sac of pregnant mice, and a potential effect on embryo growth. Nanotoxicology 10:654-661.	$AgNO_3$ AgNP (10 nm)	i.v.	Mouse (♀)	GD 7, 8, 9	□	Multiple tissues / Placenta	nc	Whole embryofetal Ag level.
Charehsaz et al., 2016	Effects of developmental exposure to silver in ionic and nanoparticle form: A study in rats. Daru. 6;24: 24.	$AgNO_3$ AgNP (55 nm)	Oral	Rat	GD 7-20	□□ Ag^+ □ to □□ NP	Multiple tissues (not placenta)	~0.04%	TD: Blood, liver, kidney, lung, **brain**. nd: **lymphoid tissues, bone marrow**.
Campagnolo et al., 2017	Silver nanoparticles inhaled during pregnancy reach and affect the placenta and the foetus. Nanotoxicology 11: 687-698.	AgNP (20 nm)	Inhalation (nose-only)	Mouse (♀)	GD 1-15	[Note 4]	Multiple tissues / Placenta	Not designated as a comparator study.	Whole embryofetal Ag level.
Paul et al., 2017	Pulmonary exposure to metallic nanomaterials during pregnancy irreversibly impairs lung development of the offspring. Nanotoxicology 11: 484-495.	AgNP	Intra-tracheal instillation	Mouse (♀)	GD 1-18 (1 dose / week)	□	Placenta	Not designated as a comparator study.	Quantitation limited to only lung Ag levels.
Fennell et al., 2017	Disposition of intravenously or orally administered silver nanoparticles in pregnant rats and the effect on the biochemical profile in urine. J Appl Toxicol. 37: 530–544.	AgAc AgNP (20; 110 nm)	Oral i.v.	Rat (♀)	GD 18	□	Multiple tissues / Placenta	0.02 – 0.05% (p.o.) 0.26 – 0.53% (i.v.)	Whole embryofetal Ag level.
Vidmar et al., 2018	Translocation of silver nanoparticles in the ex vivo human placenta perfusion model characterized by single particle ICP-MS. Nanoscale 10: 11980.	$AgNO_3$ AgNP (~7 nm; ~15 nm)	Direct perfusion	Ex vivo human placenta	N/A	N/A	N/A	~0.02–0.06%	Not applicable.

Note 1: Based on total Ag mass concentrations and expressed as the percentage of the Ag dose administered to dams.

Note 4: Animals were exposed to an atmosphere containing 3.80×10^7 particles / cm^{-3}; at a mass concentration of 640 µg/m³.