OXFORD
SOT | Society of Toxicology
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TOXICOLOGICAL SCIENCES, 174(2), 2020, 311–325

doi: 10.1093/toxsci/kfz249
Advance Access Publication Date: February 14, 2020
Research Article

Bioelution, Bioavailability, and Toxicity of Cobalt Compounds Correlate

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ABSTRACT

Based on the wide use of cobalt substances in a range of important technologies, it has become important to predict the toxicological properties of new or lesser-studied substances as accurately as possible. We studied a group of 6 cobalt substances with inorganic ligands, which were tested for their bioaccessibility (surrogate measure of bioavailability) through *in vitro* bioelution in simulated gastric and intestinal fluids. Representatives of the group also underwent *in vivo* blood kinetics and mass balance tests, and both oral acute and repeated dose toxicity (RDT) testing. We were able to show a good correlation between high *in vitro* bioaccessibility with high *in vivo* bioavailability and subsequent high *in vivo* toxicity; consequently, low *in vitro* bioaccessibility correlated well with low *in vivo* bioavailability and low *in vivo* toxicity. *In vitro* bioelution in simulated gastric fluid was the most precise predictor of the difference in the oral RDT lowest observed adverse effect levels of 2 compounds representing the highly and poorly bioaccessible subset of substances. The 2 compounds cobalt dichloride hexahydrate and tricobalt tetraoxide differed by a factor of 440 in their *in vitro* bioaccessibility and by a factor of 310 in their RDT lowest observed adverse effect level. In summary, this set of studies shows that solubility, specifically *in vitro* bioelution in simulated gastric fluid, is a good, yet conservative, predictor of *in vivo* bioavailability and oral systemic toxicity of inorganic cobalt substances. Bioelution data are therefore an invaluable tool for grouping and read across of cobalt substances for hazard and risk assessment.

Key words: metal(s); inorganics(s); read across; grouping; 3Rs; bioaccessibility.

Cobalt (Co) occurs naturally as a mineral and is present in more than 100 natural or man-made compounds, many of which are crucial in a wide range of "high-tech" applications (eg, catalysts or batteries). At trace levels, Co is present ubiquitously. Diet, eg, fish, green leafy vegetables, and cereals (Hokin *et al.*, 2004b), is the main source of cobalt for humans; intakes range from 3 to 82 μg Co/day in different regions of the world (Hokin *et al.*, 2004a).

Cobalt is involved in mammalian metabolism by sensing oxygen deficiency in animal cells by modulating transcriptional activator hypoxia-inducible factor, stimulating erythropoietin production and increasing erythropoiesis (Saxena *et al.*, 2012; Shrivastava *et al.*, 2008; Yuan *et al.*, 2001). Co ion is an essential nutrient in several species, and consequently, Co salts are used as nutritive supplements in feedstock and pharmaceutical industries. Co forms the core of vitamin B12, an essential nutrient for humans. In this form, it is required for the production of red blood cell (RBC), the prevention of pernicious anemia, in the formation of DNA, the synthesis of fatty acids and in energy metabolism (O'Leary and Samman, 2010).

Co ion absorption occurs in the duodenum and proximal jejunum and is facilitated by Divalent Metal Transporter 1, a protein also responsible for the absorption of other divalent metal

cations including iron and zinc (Knöpfel *et al.*, 2005). Vitamin B12 is absorbed in the terminal ileum by a complex process of protein binding and endocytosis (Kozyraki and Cases, 2013). In humans, approximately 4% of Co in serum is predicted to occur as free ionic Co^{2+}, the rest being bound to serum proteins, primarily albumin (Kerger *et al.*, 2013a,b). Following oral administration, unabsorbed Co from Co dichloride hexahydrate is primarily excreted via feces, (amounting to 72 ± 8%), whereas absorbed Co is excreted via urine (up to 21 ± 4%), and to a lesser extent (around 1%), in the feces via the bile (Kirchgessner *et al.*, 1994).

Water-soluble Co compounds have been administered in repeated dose studies and shown to various effects in rodents. Hematological effects were reported in a study of rats administered $CoCl_2$ in daily dosing of 12.5 mg Co/kg/day for 7 days (Shrivastava *et al.*, 2010). Degenerative cardiomyopathy was produced in rats given 100 mg Co/kg followed by 26 mg Co/kg/day for 8 weeks (Grice *et al.*, 1969). High doses in rodents (23, 42, or 72 mg Co/kg/day) generated effects on male reproductive organs such as decreased testes weight and sperm count (Anderson *et al.*, 1992; Elbethieha *et al.*, 2008; Pedigo *et al.*, 1988).

In humans, ingestion of soluble Co salts at elevated doses has been shown to produce effects on the hematologic, cardiovascular, as well as the endocrine system. Beer containing a cobalt foam stabilizer was associated with a specific and severe cardiomyopathy in a study of heavy beer drinkers (Alexander, 1972; Morin *et al.*, 1971). However, the interpretation was complicated by an incomplete description of the nutritional status of the exposed population. A study by Kesteloot *et al.* (1968) found that well-nourished drinkers did not experience the same cardiac effects despite similar ingested Co doses, suggesting that confounding factors of chronic alcoholism (malnourishment, underlying cardiac, or liver disease) may have predisposed the original cohort. Cardiomyopathy has also been reported in patients on dialysis for end-stage renal disease (Curtis *et al.*, 1976; Kriss *et al.*, 1955; Manifold *et al.*, 1978), where again underlying disease may impair a healthy response to high-dose exposure.

$CoCl_2$ was used to treat anemia in the 1950s, as it was known to increase hemoglobin (HGB) and RBC counts. A study of children with sickle cell anemia receiving 1.4–1.8 mg Co/kg/day demonstrated a decrease in iodine uptake and the development of goiters (Gross *et al.*, 1955; Kriss *et al.*, 1955). Children without sickle cell disease receiving 0.45–2.7 mg Co/kg/day for 10 weeks developed decreased iodine uptake at 5 weeks (2 of 5 children given the highest dose), while the other 13 children showed no thyroid effect after 10 weeks of dosing. The thyroid effects were reversible in both studies.

The similarities in consequences of high-dose exposure in animal studies serve as an indicator of potential health effect in humans, nevertheless these studies have focused on 2 soluble cobalt salts, $CoCl_2$ and $CoSO_4$ (as hydrated forms). The potential hazards of many cobalt compounds still need to be understood. The less water-soluble Co compounds are data-poor in comparison with the highly soluble Co inorganic salts.

The most fundamental method of differentiation of potential physiologic behavior between inorganic substances of the same metal has historically been water solubility. Water solubility does however not reflect the differences in pH along the gastrointestinal tract which vary significantly, from pH 6.2 to 7.6 in the mouth (Baliga *et al.*, 2013), to 1.5 to 3.5 in the stomach lumen, and rising to 7 to 9 in the jejunum.

Bioelution (the measurement of the release of metal ions from metal substances in artificial fluids that are surrogates for biological fluids) is a tool to identify the bioaccessibility of substances as an estimate of bioavailability of the active component of metal compounds—the metal ion. Bioaccessibility is seen as a conservative predictor of *in vivo* behavior as it describes the maximally absorbable mass of metal ion, whereas not all will be absorbed or induce damage, as effects will depend on, eg, dose or speciation (Henderson *et al.*, 2014). For most metals, ion release from a compound is maximized in acidic pH. Therefore, gastric bioelution testing has been proposed as the critical step in predictive testing for *in vivo* bioavailability, as it indicates the amount of ion that is at maximum available for absorption in the intestine. Bioelution testing, as a measure of bioavailability, can be used to read across toxicity information from data-rich members of a group of compounds ("source substances") to data-poor group members, the so-called "target substances" (Henderson *et al.*, 2012).

This paper describes a tiered approach toward the identification of the repeated dose oral toxicological hazard for a large category of related metal substances. The comparison of results from bioelution, acute oral toxicity, mass balance testing, toxicokinetic studies, and repeated dose toxicity (RDT) have contributed to the identification of a sound read-across approach and a reduction in the need for extensive animal testing.

MATERIALS AND METHODS

Materials

Tested substances

Names Used Synonymously in This Study

Substance	Molecular formula	Abbreviated formula	CAS no.	EC no.
Cobalt dichloride hexahydrate	$CoCl_2.6H_20$	$CoCl_2$	7791-13-1	231-589-4
Cobalt sulfate heptahydrate	$CoSO_4.7H_2O$	$CoSO_4$	10026-24-1	233-334-2
Cobalt sulfide	CoS	CoS	1317-42-6	215-273-3
Tricobalt tetraoxide	Co_3O_4	Co_3O_4	1308-06-1	215-157-2
Cobalt oxyhydroxide	CoOOH	CoOOH	12016-80-7	234-614-7
Cobalt lithium oxide	$CoLiO_2$	$CoLiO_2$	12190-79-3	235-362-0

Bioelution Assays

The conduct of the study was based on testing of metal substances for solubility in artificial biological fluids as described previously (Brock and Stopford, 2003; Stopford *et al.*, 2003).

In testing for gastric (pH 1.5) and intestinal (pH 7.4 ± 0.2) bioelution, each extraction was performed using 0.1 g of sample in 50 ml of the respective fluid. Samples were placed into 250 ml Erlenmeyer flasks, the relevant fluid was added and pH was checked for each solution and adjusted if necessary with 2N HCl. The flasks were shaken for 2 (gastric) or 5, 24, and 72 h (intestinal) at 37°C. The solutions were filtered through a 0.45 μm filter and the pH was verified. For bioelution in intestinal fluid, 5% CO_2 in nitrogen was bubbled through the solution at a rate of 50 cc/min to maintain a constant pH.

The 2 h extraction time was chosen based on gastric emptying half time from 18 min (Tomlin *et al.*, 1993) to 1.2 ± 0.3 h (Read *et al.*, 1986). Extractions in intestinal fluid were conducted

at 5, 24, and 72 h, based on an intestinal transit time of 4 ± 1.4 h in humans (Read *et al.*, 1986). Reported are only the 24-h results.

Analysis

The dissolved cobalt ion concentrations in the test item vessels and the blank control were determined using ICP-MS. A detection limit for cobalt was determined as the rounded value of 3 times the standard deviation (SD) of all blank measurements ($N = 6$) sampled.

Acute Oral Toxicity Testing

All studies were GLP and OECD-compliant; Co_3O_4 and CoS were tested according to the OECD 401 guideline and CoOOH and $CoLiO_2$ were tested according to the OECD 425 guideline. In addition, previously published data for $CoCl_2$ and $CoSO_4$ are used in the acute toxicity database.

Plasma Toxicokinetic Studies

These studies were conducted according to OECD 417 and under GLP. Sixty-day-old male and female Crl:CD(SD) rats (obtained from Charles River Laboratories, Germany) were kept under standard conditions in groups of 3 animals (same sex) with 12/12 h dark-light cycle. Feed (ssniff R/M-H V) and water were given *ad libitum*. The animals were randomly assigned to groups of 10 males and 10 females each. The animals in the different groups received

1. a single intravenous injection of 0.1 mg $CoCl_2$/kg bw (0.0248 mg Co/kg bw) in 0.9% NaCl (internal comparison used in each of 2 separate studies for comparison of oral doses of $CoCl_2$ and Co_3O_4, and cobalt lithium dioxide and CoS),
2. $CoCl_2$ (oral dose of 10 mg/kg bw [2.48 mg Co/kg bw]),
3. Co_3O_4,
4. $CoLiO_2$, or
5. CoS, the substances (3)–(5) each at an oral dose of 300 mg substance/kg bw/day, corresponding to 220 mg/kg, 180 mg/kg, and 194 mg Co/kg, respectively.

All oral bolus doses were given as a single oral gavage in 0.5% HPMC (hydroxypropyl methylcellulose), at dose levels not causing any acute or local toxicity or irritation.

The test item formulations were administered at a constant administration volume of 5 ml/kg bw on the administration day. Animals were allocated to the 4 test groups by means of a computer-generated randomization program. Citrate plasma (100 µl) was collected from the *vena jugularis* of all animals under isoflurane anesthesia at 0, 1, 4, 12 h after dosing in 1 subset, and after 0.5, 2, 8, 24 h in another subset of animals.

Plasma samples were digested by addition of concentrated nitric acid (69%) followed by microwave digestion (Ultra Clave, MLS, Leutkirch, Germany; step 1: 25 min up to 220°C; step 2: 30 min hold 220°C). Analysis of liquid certified reference material (TM-25.4 [lot no. 0914], TMDA-53.3 [lot no. 0914], TM-26.4 [lot no. 0615], and TMDA-52.4 [lot no. 1115], Environment Canada), recalibration standards (Merck Certipur Cobalt ICP standard 1000 mg/l lot no. HC41722713) and quality control standards were performed by ICP-MS (Agilent 7500, Agilent Technologies, Waldbronn, Germany). No certified reference material for digestion in a biological matrix was available for lithium, thus recovery of cobalt was used as validation of the digestion. The limit of detection (LOD) was calculated as 3 times the SD of calibration blank divided by the slope of the calibration curve (average of LODs from 10 independent measurements: 0.000889 µg Co/l); the limit of quantification (LOQ) was calculated as 3 times the LOD (average of LOQs from 10 independent measurements: 0.0033 µg Co/l).

Pharmacokinetic evaluation of the plasma data was performed using WinNonlin version 6.4 (Pharsight Corporation, Mountain View, California) with a noncompartment model. Elimination rate constants (K_{el}) and plasma elimination half-lives ($t_{1/2}$) were calculated by linear regression analysis of the log/linear portion of the individual plasma concentration-time curves (c = concentration, t = time). Area under the curve (AUC) values were calculated using the linear trapezoidal method and $AUC_{0-t\ last}$ was calculated according to the linear trapezoidal rule. Values below or at the LOQ were excluded from WinNonlin-calculation. The oral bioavailability of each cobalt substance is expressed as relative bioavailability, normalized to the level of administered Co dose, compared with $CoCl_2$ given IV.

Mass Balance Studies

The elimination kinetics and mass balance of cobalt excretion after single oral application via gavage of $CoCl_2$ and Co_3O_4 was studied in Crl:CD(SD) rats (obtained from Charles River Laboratories) at doses not causing any acute toxicity or irritation. Animals were randomly assigned in 2 groups of 5 males and 5 females each. One group received 10 mg/kg bw $CoCl_2$ (2.48 mg Co/kg bw), a second group received 300 mg/kg bw Co_3O_4 (220 mg Co/kg bw) in 0.5% HPMC in a total volume of 5 ml/kg bw. The third group (control) received vehicle only. After administration, the animals were kept in metabolism cages, 24-h urine and feces were collected in 3 fractions/animal.

Urine was analyzed by ICP-OES and ICP-MS (ICP-MS only 1 group) without any pretreatment. Feces were initially freeze-dried (lyophilization), followed by homogenization and microwave digestion (Ultra Clave, MLS; step 1: 25 min up to 220°C; step 2: 30 min hold 220°C) in concentrated nitric acid. Analysis of certified aqueous reference materials (TM-25.4 [lot no. 0914], TM-26.4 [lot no. 1115], TMDA-53.3 [lot no. 0914], and TMDA-54.5 [lot nos 0815 and 0316], Environment Canada), recalibration standards (Merck Certipur Cobalt ICP standard 1000 mg/l lot no. HC41722713) and quality control standards were performed by ICP-MS (Agilent 7500, Agilent Technologies) and ICP-OES (Agilent 720, Agilent Technologies). The LOD was calculated as 3 times the SD of calibration blank divided by the slope of the calibration curve; the LOQ was calculated as 3 times the LOD. Background level of Co through vitamin B12 in feed was measured in nonexposed animals (vehicle controls) and subtracted from the Co extraction levels in the test animals.

Target Organ Study

After 90-day oral exposure to 30 mg $CoCl_2$-hexahydrate/kg bw/day (7.44 mg/kg bw/day as Co) described above, 5 male and 5 female animals of the control (0 mg $CoCl_2$-hexahydrate/kg bw/day) and high-dose (30 mg $CoCl_2$-hexahydrate/kg bw/day) groups were housed in metabolic cages for 24 h to collect 24-h urine and feces samples, as described in the mass balance study method description. Cobalt analysis was performed as described above (Mass balance study).

RDT Studies

All studies followed the most recent OECD 408 or 422 guidelines and were conducted under GLP.

Animals

For all studies male and female Crl:CD(SD) rats (Charles River Laboratories) aged between 52 and 65 days were used. Only healthy animals were used for all studies. Health checks were performed on the day of delivery and at first administration. Each animal was free of signs for abnormality or disease. Animals were kept at a 12/12 h dark light cycle and received feed (ssniff R/M-H V1534, ssniff Spezialdiäten GmbH, Germany) and water *ad libitum*.

For the conduct of the reproductive toxicity screen, sexually mature male and female rats were randomly paired for mating to achieve at least 8 pregnant dams per group.

Test protocols

The dose levels for these studies were selected based on available toxicological data, and on dose range-finding studies for $CoCl_2$ (not reported here; data available upon request).

For the subchronic oral RDT study (OECD 408), 100 animals (40 male and 40 female rats for the main study and 10 male and 10 female rats for the recovery study, control and highest dose only) were exposed as described below:

Tricobalt tetraoxide (purity 98%) and $CoCl_2$ (purity 99.8%), were administered orally by gavage in 0.5% HPMC once daily for 90 days at a constant volume of 5 ml/kg bw and 2 ml/kg bw, respectively. The doses used for this study were 0, 100, 300, and 1000 mg Co_3O_4/kg bw/day (0, 73.4, 220, and 734 mg Co/kg bw/day) and 0, 3, 10, and 30 mg $CoCl_2$/kg bw/day (0, 0.74, 2.48, and 7.44 mg Co/kg bw/day). The control animals received the vehicle in the same way once daily.

For the OECD 422 combined RDT study with the reproduction/developmental toxicity screening test for CoS, 80 animals (40 males and 40 females) were randomly allocated, 10 males and 10 females per group, to 1 of 4 test groups of 0 (control), 100, 300, or 1000 mg/kg bw/day (corresponding to 0, 64.8, 194, and 648 mg Co/kg bw/day). Cobalt sulfide (mixture of 86.5% Co9S8 and 13.5% CoS1.032) was administered orally by gavage in 0.5% HPMC once daily. For males, dosing began 2 weeks before mating and continued during the mating period and approximately 2 weeks post until the minimum total dosing period of 28 days was completed. For females, dosing began 2 weeks before mating and continued up to and including day 3 postpartum.

For the OECD 422 study of Co_3O_4, 80 animals (40 males and 40 females) were randomly allocated, 10 males and 10 females per group, to 1 of 4 test groups of 0 (control), 100, 300, and 1000 mgCo_3O_4/kg bw/day (corresponding to 0, 73.4, 220, and 734 mg Co/kg bw/day). Tricobalt tetraoxide (purity > 99.5%) was administered orally by gavage in 0.5% HPMC once daily for the same dosing frequency as the CoS study.

For all studies, the standard panel of clinical observations, neurological and observational screening, functional tests, hematology and clinical biochemistry, coagulation, body weight were measured and food and drinking water consumption were monitored. As additional parameters, testosterone, progesterone, and 17beta-estradiol levels were determined in all animals predose, during treatment (test day 42), at the end of test week 13 (test day 91) and at the end of the recovery period (test day 119). Furthermore, all stages of the estrus cycle were determined in all female animals predose, during study conduct (test weeks 5/6), at the end of the treatment period (test weeks 12/13) and at the end of the recovery period (test weeks 16/17). Due to the wealth of parameters measured in these studies, only those endpoints that were affected by the treatment are reported. The list of measured endpoints is available upon request.

The animals were euthanized by carbon dioxide (CO_2), exsanguinated by cutting the aorta abdominalis, weighed, dissected, and inspected macroscopically approximately 24 h after the last administration. F1 generation pups were sacrificed on lactation day 4. All tissues and organs underwent macroscopic inspection as well as histopathological examination according to the OECD 422 guideline.

Statistical Analyses

Differences in AUC between males and females were investigated by a *t* test (2-sample assuming equal variances, with an alpha value of .05), carried out in Excel (Windows 10).

For the mass balance and target organ studies, the concentrations of cobalt in the relevant biological matrix (feces, urine, or wet tissue) are presented as average and SD of 5 males and 5 females each (mass balance), or 10 males and 10 females each (target organ study), both calculated in Excel (Windows 10).

Statistical analysis of the hematological parameters in the OECD 408 studies was performed using a 1-way ANOVA with an alpha value of .05. For those endpoints where the null hypothesis (H_0) was rejected ($p < .05$), a post hoc analysis was performed by comparing each treatment group with the control group using a pairwise *t* test (2-sample assuming equal variances) with a Bonferroni corrected alpha value of .0167 (.05/3). If the comparison resulted in a $p < .01$ (rounded from 0.0167), the treatments group was labeled with *. *p* Values < .001 were labeled with **. ANOVA and *t* test were performed using Excel "data analysis" add in (Windows 10). The ANOVA results are shown in Supplementary Tables.

The evaluation of the statistical differences between the individual treatment groups and control was followed by benchmark dose modeling to determine the BMD10 dose using EPA's latest Benchmark Dose Software (BMDS 3.1) for continuous toxicity endpoints. BMD modeling was performed using a 1 standard deviation (1STD) as the benchmark response, on the hematological parameters from oral administration of $CoCl_2$ (Table 1) and of Co_3O_4 (Table 2). Data were modeled with both constant (homogenous) and nonconstant (nonhomogenous) variance models. Adequate fit is measured by a series of 4 tests. Test 1 ensures that there is a difference in effect between the modeled doses; a *p* value of .05 or less is required to reject H_0 that there is no difference in effect. Test 2 tests if the variances of the model are homogenous; if the *p* value is < .1, a homogenous model is not appropriate, and a nonhomogenous model should be considered. Test 3 ensures that the variance is appropriately modeled; the *p* value for this test should be > .1. Test 4 is a goodness of fit test; if the *p* value is > .1, then the model adequately fits the data.

The 90-day exposure studies with $CoCl_2$ and Co_3O_4 were followed by a 3-month recovery group in the control and highest dose group only. The results were analyzed by pairwise comparison (*t* test with 2-sample assuming equal variances) at an alpha value of .05 comparing control versus high dose at days 90 and 119. Comparisons yielding a $p < .05$ were labeled with *, $p < .01$ was labeled with **.

A parameter was considered unaffected by the treatment ("unchanged") either if the ANOVA did not lead to a rejection of H_0, or if the post hoc analysis did not yield any *p* value of < .01.

RESULTS

Predictive In Vitro *Testing of Cobalt Compounds*

Oral bioelution testing has been performed for 15 Co substances with an inorganic counter ion or a very short-chain organic

Table 1. Hematological Parameters From Oral Administration of $CoCl_2$

(A) Males

Males $CoCl_2$, experimental group				HGB (mmol/l)	RBC (×10E6/μl)	Reti (‰ of RBC)	PLT (×10E3/μl)	HCT (%)
At end of 90-day oral exposure	Control	$N = 10$	Mean	10.35	9.46	14.1	917	49.91
	0.74 mg Co/kg bw/day	$N = 10$	Mean	10.50	9.33	15.2	793.6	50.51
			p	.4147	.5858	.4662	.1286	.4347
			%Diff vs control	+1.4%	−1.4%	+7.8%	−13.5%	+1.2%
	2.48 mg Co/kg bw/day	$N = 10$	Mean	11.46*	10.33*	10.8	789.2	55.07*
			p	.0017	.0061	.0292	.0267	.0022
			%Diff vs control	+10.7%	+9.2%	−23.4	−13.9%	+10.3%
	7.44 mg Co/kg bw/day	$N = 10$	Mean	13.00**	11.25**	10.5	637.1**	61.98**
			p	4.8E−10	4.5E−06	.0271	1.04E−05	8.071E−09
			%Diff vs control	+25.6%	+18.9%	−25.5	−30.5%	+24.2%
Males $CoCl_2$, recovery group								
At end of 90-day oral exposure	Control	$N = 5$	Mean	10.28	9.2	20	880.4	48.26
	7.44 mg Co/kg bw/day	$N = 5$	Mean	12.74**	10.89**	15.6	727.2	58.68**
			p	.0015	.0022	.400	.223	.002
At end of 28-day recovery period	Control	$N = 5$	Mean	10.00	8.698	23.2	845.8	47.78
	7.44 mg Co/kg bw/day	$N = 5$	Mean	10.64	9.086	15.1	917.6	49.58
			p	.087	.373	.0544	.551	.334

(B) Females

Females $CoCl_2$, experimental group				HGB (mmol/l)	RBC (×10E6/μl)	Reti (‰ of RBC)	HCT (%)
At end of 90-day oral exposure	Control	$N = 10$	Mean	9.53	8.33	14.8	44.98
	0.74 mg Co/kg bw/day	$N = 10$	Mean	9.62	8.38	12	45.37
			p	.4453	.7432	.1951	.5589
			%Diff vs control	+0.9%	+0.6%	−18.9%	
	2.48 mg Co/kg bw/day	$N = 10$	Mean	9.56	8.11	19.7	45.19
			p	.8877	.4580	.1685	.8327
			%Diff vs control	+0.3%	−2.6%	+33.1%	
	7.44 mg Co/kg bw/day	$N = 10$	Mean	10.81**	9.15*	15.4	51.14*
			p	.0005	.0088	.7781	.0015
			%Diff vs control	+13.4%	+9.8%	+4.1%	
Females $CoCl_2$, recovery group							
At end of 90-day oral exposure	Control	$N = 5$	Mean	9.62	8.458	15.4	45.3
	7.44 mg Co/kg bw/day	$N = 5$	Mean	11.1**	9.648*	15.6	52.3**
			p	.0017	.030	.933	.0025
At end of 28-day recovery period	Control	$N = 5$	Mean	9.52	7.984	16.8	44.64
	7.44 mg Co/kg bw/day	$N = 5$	Mean	9.56	8.01	15	45.02
			p	.936	.948	.232	.841

$CoCl_2$ exposure: hematological parameters male and female rats on test days 91/92 and on test day 119, following a 28-day recovery period (only control and high-dose group). Reported are only those endpoints that displayed a change.

All treatment groups and control group were compared by 1-way ANOVA (alpha value .05). In those cases where $p \leq .05$, the ANOVA was followed by pairwise comparison between each treatment group with the control group (t test, 2-sample assuming equal variances) with a Bonferroni correction. In the "experimental group" data, $^{*}p < .01$, $^{**}p < .001$.

Recovery groups: only those endpoints displaying an effect were analyzed for reversibility. The highest dose treatment group was compared by pairwise comparison with the concurrent control group at days 91/92 (end of treatment period) and at day 119, following a 3-month washout period (t test, 2-sample assuming equal variances) with an alpha value of .05. For the "recovery group" data, $^{*}p < .05$, $^{**}p < .01$.

counter ion (acetate and propionate, ≤ 3 carbon atoms), referred to as "inorganic cobalt substances."

Bioelution Testing

The results of the solubility of 6 Co substances, $CoCl_2$, $CoSO_4$, CoOOH, $CoLiO_2$, CoS, and Co_3O_4 in the biologically relevant fluids, simulated gastric fluid (pH 1.5) and simulated intestinal fluid (pH 7.4), are presented in Table 3. The inorganic salts $CoCl_2$ and $CoSO_4$ are very soluble in both acidic and slightly basic fluids. Cobalt sulfide, Co_3O_4, $CoLiO_2$, and CoOOH are also inorganic cobalt salts, yet are poorly soluble in both gastric and intestinal fluids. In contrast, cobalt metal powder is very soluble in gastric fluid, however poorly soluble in intestinal fluid. (Available data for Co metal powder include water solubility, bioelution in gastric and intestinal fluids, acute oral toxicity, and a RDT 422 with range finder. However, Co metal powder is highly irritating in the stomach of animals after dosing beyond 14 days. In the 422 study there were excessive amounts of deaths with aspiration and potential misdosing. Qualitatively, local irritation prohibits testing of Co metal powder *in vivo* (peroral route) at RDT testing beyond

Table 2. Hematological Parameters From Oral Administration of Co_3O_4

(A) Males

Males Co_3O_4, experimental group				HGB (mmol/l)	RBC (×10E6/µl)	PLT (×10E3/µl)	HCT (%)
At end of 90-day oral exposure	Control	N = 10	Mean	10.07	9.20	860.4	48.28
	73.4 mg Co/kg bw/day	N = 10	Mean	10.29	9.30	896.5	49.53
			p	.2338	.6597	.5607	.1940
			%Diff vs control	+2.2%	+1.1%	+4.2%	+2.6%
	220 mg Co/kg bw/day	N = 10	Mean	11.03*	10.08*	796.1	52.73*
			p	.0035	.0080	.2804	.0071
			%Diff vs control	+9.5%	+9.6%	+7.5%	+9.2%
	734 mg Co/kg bw/day	N = 10	Mean	12.63**	11.29**	573.9**	59.97**
			p	8.67E−10	9.56E−07	.0005	2.02E−9
			%Diff vs control	+25.4%	+22.7%	+33.3%	+24.2%
Males Co_3O_4, recovery group							
At end of 90-day oral exposure	Control	N = 5	Mean	10.1	9.12	932.4	48.72
	734 mg Co/kg bw/day	N = 5	Mean	12.78**	11.3**	645.6**	60.96**
			p	2.4E−06	7.1E−05	.0085	9.1E−06
At end of 28-day recovery period	Control	N = 5	Mean	9.72	8.61	995.2	46.04
	734 mg Co/kg bw/day	N = 5	Mean	10.82	9.47	882.4	50.38
			p	.075	.052	.285	.126

(B) Females

Summary

Females Co_3O_4, experimental group				HGB (mmol/l)	RBC (×10E6/µl)	HCT (%)
At end of 90-day oral exposure	Control	N = 10	Mean	9.53	8.28	45.01
	73.4 mg Co/kg bw/day	N = 10	Mean	9.51	8.17	44.76
			p	.8603	.4158	.6754
			%Diff vs control	+0.2%	+1.3%	−0.6%
	220 mg Co/kg bw/day	N = 10	Mean	10.09*	8.74	46.99
			p	.0031	.0139	.0366
			%Diff vs control	+5.9%	+5.6%	+4.4%
	734 mg Co/kg bw/day	N = 10	Mean	11.09**	9.35**	51.27**
			p	2.39E−06	.00014	1.52E−05
			%Diff vs control	+16.4%	+12.9%	+13.9%
Females Co_3O_4, recovery group						
At end of 90-day oral exposure	Control	N = 5	Mean	9.82	8.56	45.88
	734 mg Co/kg bw/day	N = 5	Mean	10.86*	9.12	50.76*
			p	.032	.17	.013
At end of 28-day recovery period	Control	N = 5	Mean	9.54	7.764	43.7
	734 mg Co/kg bw/day	N = 5	Mean	9.28	7.56	40.76
			p	.450	.573	.530

Co_3O_4 exposure: hematological parameters male and female rats on test days 91/92 and on test day 119, following a 28-day recovery period (only control and high-dose group). Reported are only those endpoints that displayed a change.

All treatment groups and control group were compared by 1-way ANOVA (alpha value .05). In those cases where $p \leq .05$, the ANOVA was followed by pairwise comparison between each treatment group with the control group (*t* test, 2-sample assuming equal variances) with a Bonferroni correction. In the "experimental group" data, $^{*}p < .01$, $^{**}p < .001$.

Recovery groups: only those endpoints displaying an effect were analyzed for reversibility. The highest dose treatment group was compared by pairwise comparison with the concurrent control group at days 91/92 (end of treatment period) and at day 119, following a 3-month washout period (*t* test, 2-sample assuming equal variances) with an alpha value of .05. For the "recovery group" data, $^{*}p < .05$, $^{**}p < .01$.

14 days. Data are available upon request but are not further discussed here.) The solubilities in gastric fluid as well as in intestinal fluid of these inorganic cobalt substances vary by over 1000-fold in intestinal- and up to 100-fold in gastric fluid. Results for all 15 substances are given in Supplementary Table 1.

Acute Oral Toxicity Testing

Acute oral toxicity testing has been performed for the majority of the cobalt substances and the results of these tests for the 6 inorganic cobalt substances also appear in Table 3. The gastric-soluble inorganic compounds $CoCl_2$ and $CoSO_4$ demonstrate acute oral toxicity at LD50s below 1000 mg/kg bw of administered compound. The least soluble compounds CoS and Co_3O_4 have acute oral toxicity values exceeding 11 000 mg/kg bw of administered compound. Co oxide hydroxide and Co lithium dioxide, poorly soluble compounds with a somewhat higher gastric solubility, have unbound acute oral toxicity values exceeding 5000 mg/kg bw. The difference in acute oral toxicity between the soluble salts represented by $CoCl_2$ and $CoSO_4$ versus the insoluble compounds CoS and Co_3O_4 is up to approximately 100-fold,

Table 3. Bioelution, Acute, and RDT of Selected Cobalt Compounds

Compounds	Co Dichloride Hexahydrate	Co Sulfate Heptahydrate	Co Oxide Hydroxide	Co Lithium Dioxide	Co Sulfide	Tricobalt Tetraoxide
In vitro						
Bioelution of Co in gastric fluid (% release), 2 h	98.5	99.7	0.85	5.9	0.58	0.2
Bioelution of Co in gastric fluid (µg Co/g sample), 2 h	243 000	214 720	4460	89 840	3820	1460
Bioelution of Co in intestinal fluid (% release)	79 (2 h)	64 (24 h)	0.06 (24 h)	0.02 (24 h)	0.33 (24 h)	0.05 (24 h)
Bioelution of Co in intestinal fluid (µg Co/g sample)	198 700 (2 h)	139 000 (24 h)	370 (24 h)	134 (24 h)	2180 (24 h)	385 (24 h)
In vivo, acute exposures						
Acute LD50 (mg substance/kg bw)	766 Speijers *et al.* (1982)	310 Domingo *et al.* (1984)	> 5000	> 5000	> 11 000	> 11 000
Acute LD50 (mg Co/kg bw)	190	65	> 3205	> 3011	> 7124	> 8076
Bioavailability calculated from AUC as % of $CoCl_2$ IV AUC, average of m + f	Absolute bioavailability 9.3%			Relative bioavailability 0.28%	Relative bioavailability 0.08%	Relative bioavailability 0.08%

Table 4. Pharmacokinetic Parameters for Several Cobalt Compounds

	Pharmacokinetic Parameters of Cobalt Dichloride Hexahydrate, Tricobalt Tetraoxide, Cobalt Sulfide, and Cobalt Lithium Dioxide in Rats (Noncompartmental Analysis)[a]							
Test item	Dose level [mg test item/kg]	Dose level [mg Co/kg]	Sex	C_{max}[b] [µg/l]	$t_{1/2}$ [h]	K_{el} [1/h]	$AUC_{0-t\ last}$/cobalt dose [(h·µg/l)/(mg/kg)]	Bioavailability [%]
$CoCl_2.6H_2O$	0.1 (IV)	0.0248	M	1.05	88.2	0.0079	293.6	Defined as 100
			F	0.40	40.7	0.0170	117.3	Defined as 100
$CoCl_2.6H_2O$	10 (PO)	2.48	M	2.51	14.2	0.0489	20.0	6.81 (absolute)
			F	2.61	13.7	0.0508	13.7	11.7 (absolute)
Co_3O_4	300 (PO)	214	M	2.08	17.3	0.0402	0.18	0.06 (relative)
			F	1.10	16.1	0.0430	0.12	0.1 (relative)
$CoCl_2.6H_2O$	0.1 (IV)	0.0248	M	0.42	13.9	0.0499	124.7	Defined as 100
			F	0.42	10.1	0.0686	114.7	Defined as 100
CoS	300 (PO)	194	M	2.01	16.8	0.0413	0.10	0.08 (relative)
			F	2.01	14.9	0.0464	0.10	0.09 (relative)
$CoLiO_2$	300 (PO)	180	M	3.45	13.0	0.0535	0.37	0.30 (relative)
			F	2.88	12.20	0.0568	0.29	0.25 (relative)

[a]The values presented in this table are rounded for reasons of better readability.
[b]Values obtained from plasma analysis (data provided by Fraunhofer-IME), all other values calculated by pharmacokinetic evaluation performed by LPT.
Abbreviations: M, male; F, female; IV, intravenous; PO, per os.

although this cannot be calculated as the acute toxicity value of the insoluble compounds is unbounded.

A trend of 2 categories in the cobalt family, namely, "soluble and potentially bioavailable" and "poorly soluble and not predicted to be bioavailable" emerged. Based on these 2 categories, toxicokinetic (TK) studies were conducted to measure *in vivo* bioavailability of representatives of each group.

Plasma TK Studies

Blood plasma kinetic studies were conducted in rats to obtain estimates of basic TK parameters (C_{max}, half-life, and AUC) for the moiety of interest, the Co ion (Table 4). Co ion was provided by 4 different cobalt compounds:$CoCl_2$, Co_3O_4, $CoLiO_2$, and CoS. The aim of the study was to obtain comparative estimates of Co bioavailability from different compounds in an abbreviated study design, to confirm or reject difference seen in the bioelution studies. As a reference value, $CoCl_2$ was also given by the IV route of exposure in 0.9% NaCl, assuming to represent 100% bioavailability, and also followed for the same time course. Expression of the ratio of the $AUC_{0-t\ last}$ of each orally administered compound to the $AUC_{0-t\ last}$ of $CoCl_2$ administered IV as a percentage gives an indication of the "% bioavailability," which

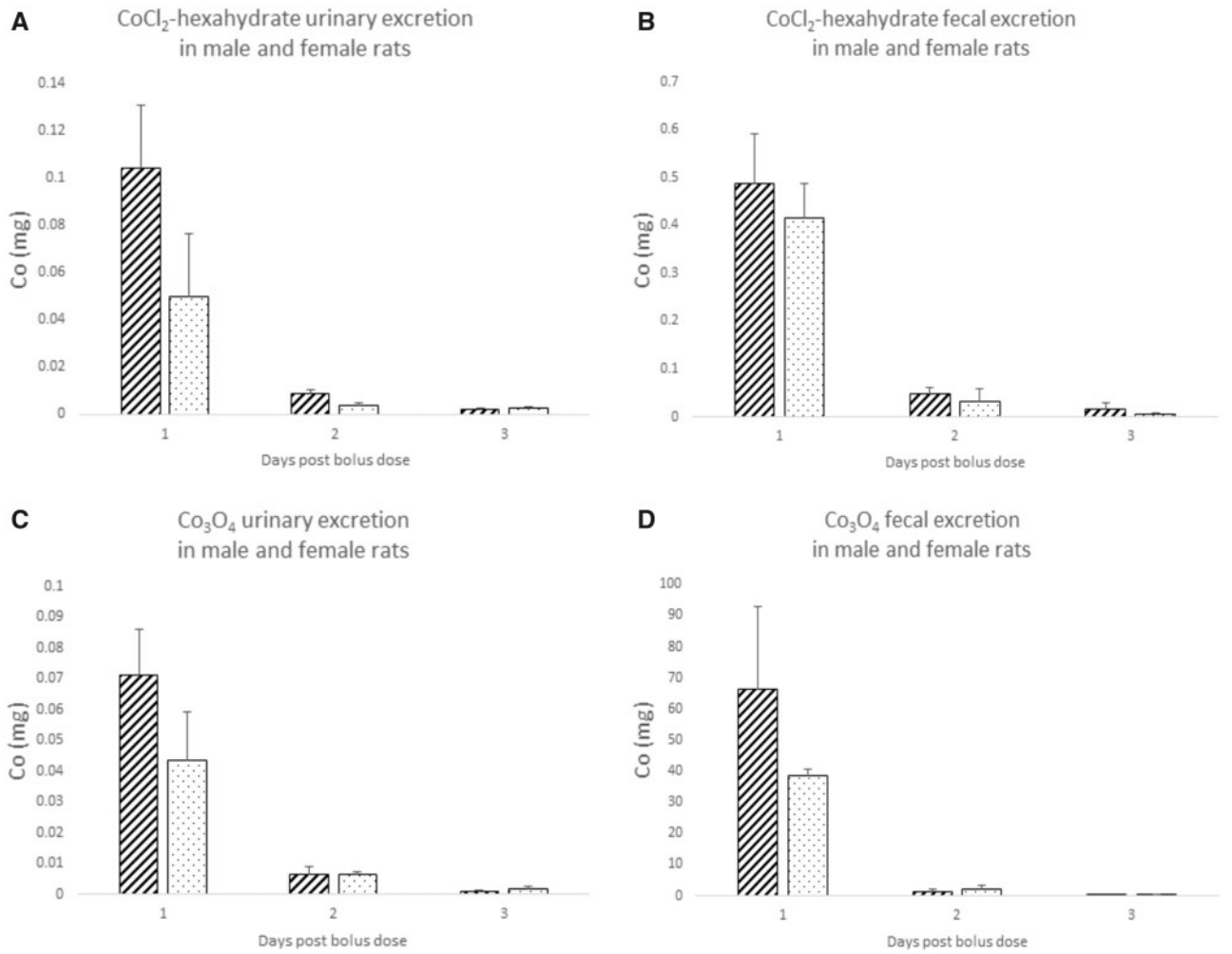

Figure 1. A, Urinary excretion of Co supplied as cobalt dichloride hexahydrate, measured during 72-h postbolus. Absolute cobalt content measured in urine of male (striped) and female (dotted) rats after receiving cobalt dichloride hexahydrate (2.48 mg Co/kg bw) in a single oral bolus dose. Data shown are means ($n = 10$) ± SD. B, Fecal excretion of Co supplied as cobalt dichloride hexahydrate, measured during 72-h postbolus. Absolute cobalt content measured in feces of male (striped) and female (dotted) rats after receiving cobalt dichloride hexahydrate (2.48 mg Co/kg bw) in a single oral bolus dose. Data shown are means ($n = 10$) ± SD. C, Urinary excretion of Co supplied as tricobalt tetraoxide, measured during 72-h postbolus. Absolute cobalt content measured in urine of male (striped) and female (dotted) rats after receiving tricobalt tetraoxide (220 mg Co/kg bw) in a single oral bolus dose. Data shown are means ($n = 10$) ±SD. D, Fecal excretion of Co supplied as tricobalt tetraoxide, measured during 72-h postbolus. Absolute cobalt content measured in feces of male (striped) and female (dotted) rats after receiving tricobalt tetraoxide (220 mg Co/kg bw) in a single oral bolus dose. Data shown are means ($n = 10$) ±SD.

can be used as a comparison of oral *in vivo* bioavailability between the different substances. The oral bioavailability of $CoCl_2$ was calculated to be approximately 7% and 12% in males and females, respectively. Co lithium dioxide had an oral bioavailability of 0.3% (males) and 0.25% (females), and the bioavailability of Co_3O_4 and CoS was below 0.1% without a pronounced difference between males and females. When considering each test substance and each group (sex) separately, the following can be observed for the orally applied substances: the maximal plasma concentration of Co achieved during the 72 h of observation (C_{max}) varies about 30% between the different compounds and sexes, with an average of 2.3 µl Co/l and a minimum of 1.1 and a maximum of 3.45 µl Co/l. The Elimination Constant (K_{el}) is relatively constant at 0.04 (1/h), with a variation of 12.5%. The half-life $t_{1/2}$ also shows small variation between the orally applied substances (average of 14.8 h with a variation of 12.4%).

Large differences are observed in the AUCs, as evident from the raw data ($AUC_{0-\ t\ last}$), resulting in the large differences in the calculated oral bioavailability of the different compounds shown in Table 4. Pronounced differences in bioavailability between sexes were observed only for $CoCl_2$ following oral exposure (6.81% in males vs 11.7% in females), however this was not statistically significant.

Mass Balance Testing

Based on the findings of Table 4, we then investigated the elimination kinetics and mass balance of cobalt excretion after single oral application via gavage. Two differently soluble cobalt substances ($CoCl_2$ and Co_3O_4) were tested at doses not causing any acute toxicity or irritation. The results are shown in Figure 1 as absolute cobalt amounts excreted in urine and feces for each of the first 3 days following application. The overall mass balance is presented in Table 5 as percent (%) of recovered dose 72-h postapplication. The cobalt content from both substances appears to be excreted predominantly during the first 24-h postgavage. The concentrations in both urine and feces decrease on day 2 by about 10-fold compared with day 1, with a further decrease on day 3 of the study. Mass balance was less than ±20%

Table 5. Co Excretion During 72 h Following Application of Bolus Dose of Several Co Compounds, Expressed as % of Co Applied

Co Compound	Sex	Urinary Excretion (%)	Fecal Excretion (%)	Sum of Urinary and Fecal Excretion (%)
$CoCl_2.6H_2O$	Males	17.1	82.4	99.5
	Females	12.2	98.5	110.7
Co_3O_4	Males	0.13	116.4	116.5
	Females	0.12	95.2	95.3

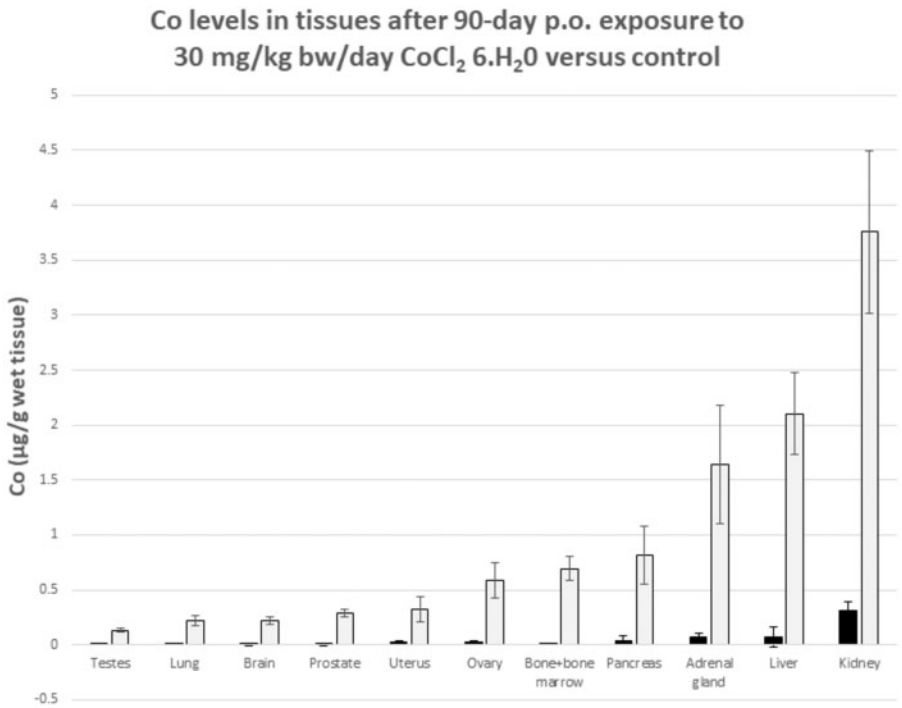

Figure 2. Tissue distribution of cobalt dichloride hexahydrate after a 90-day repeated dose (oral) exposure versus control animals. Cobalt levels in tissues after repeated oral bolus doses of cobalt dichloride hexahydrate (7.4 mg Co/kg bw/day), open bars, compared with sham controls receiving vehicle only, filled bars. Data shown are means ($n = 10$) ±SD. Data are given in table format as Supplementary File.

of the administered dose, which is within an acceptable range. Fecal excretion was the predominant route, with 80% ($CoCl_2$) to 99% (Co_3O_4) excreted via the feces, and the remainder of the dose being excreted via urine.

In Supplementary Tables 2 and 3, urinary excretion is shown as µg Co/l and µg Co/µmol creatinine (creat); and fecal excretion as µg Co/g. The SD of the average Co concentration in urine expressed by volume was compared with the matching concentration corrected by creatinine. Of the 12 pairs (calculated averages and SDs expressed as %), the SD of the creatinine corrected values was smaller in 10 cases. This indicates that the creatinine correction reduces the variability compared with Co concentration in urine expressed as µg Co/l.

Tissue distribution of cobalt after subchronic exposure. Co concentrations in tissues of the control versus exposed animals (90-day exposure to 7.44 mg Co/kg bw/day as $CoCl_2$) are shown in Figure 2 and Supplementary Table 4. For most tissues and organs, the increases in Co concentration are relatively uniform, all at an elevation of around 20-fold versus control animals' tissue Co concentrations. The highest increase in tissue Co concentration versus control animals is observed in the bone marrow, where the Co concentration had multiplied by over 100-fold. The highest absolute Co concentrations following exposure were observed in the liver and kidney.

RDT Testing

Repeated dose toxicity testing was performed on 1 or more representative substances from each of the categories from the inorganic cobalt compound family. Gastric-soluble inorganic cobalt compounds were represented by $CoCl_2$, and gastric-poorly soluble inorganic compounds by Co_3O_4 and CoS.

Cobalt dichloride hexahydrate: Subchronic RDT study with reproductive toxicity screening (OECD 408)

None of the animals treated with any dose of $CoCl_2$ died prematurely. No test item-related changes were noted for neurological screening, behavior, external appearance, body posture, or movement and coordination capabilities, food and drinking water consumption, feces, estrous cycle urinalysis, hormone levels, ophthalmoscopic examination, macroscopic postmortem findings, or organs weights including testes and prostate. There were no macroscopic or histopathological findings in heart or thyroid at any dose.

The body weight of the male and female animals treated with the highest dosage of 7.44 mg Co (as $CoCl_2$)/kg bw/day was reduced by 5% to 14% from test day 8 onwards and by 5% to 10% from test day 29 onwards, respectively, compared with the control group. The body weight at autopsy was reduced by 11% (males) and 9% (females), respectively. At the end of the 4-week recovery period (test day 118), the body weight of the male and

Table 6. Ninety-day RDT Studies (Including Reproductive Toxicity Screening) With Cobalt Dichloride Hexahydrate and Tricobalt Tetraoxide (OECD 408)

	Cobalt Dichloride Hexahydrate	Tricobalt Tetraoxide
Doses (as cobalt)	0.74, 2.5, 7.5 mg Co/kg bw	73.4, 220, 734 mg Co/kg bw
NOAEL	0.74 mg Co/kg bw/day Based on onset of hematological effects at the next highest dose	220 mg Co/kg bw/day Based on nonadverse increases in RBC, HGB, HCT
LOAEL (systemic)	2.5 mg Co/kg bw/day Based on increase in HGB, RBC, HCT and decrease in Reti and PLT, and erythroid hyperplasia in bone marrow	734 mg Co/kg bw/day Based on significant hematological changes (RBC, HGB, HCT)
NOAEL (reproductive)	> 7.5 mg Co/kg bw/day Based on the complete absence of findings on any reproductive parameter	> 734 mg Co/kg bw/day Based on the complete absence of findings on any reproductive parameter

Abbreviations: NOAELs, no observed adverse effect levels; LOAELs, lowest observed adverse effect levels.

female animals exposed to the highest dose was still reduced by 17% or by 13%, respectively, compared with the control group.

No test item-related changes in hematological parameters were noted for the male and female animals treated with 0.74 mg Co/kg bw/day at the end of the treatment period. Male animals showed a statistically significant increase in HGB, total RBCs, and hematocrit (HCT) at 2.48 ($p < .01$) and 7.44 ($p < .001$) mg Co/kg bw/day. At the same doses, there was also a decrease in reticulocytes (Reti) and platelet (PLT) counts, both of which were reduced by more than 10%, without statistical significance ($p < .01$) at 2.48 mg Co/kg bw/day, and with only the reduction in PLT resulting in a statistically significant decrease ($p < .001$) at 7.44 mg Co/kg bw/day. Females had less severe effects, with an increase in HGB ($p < .001$), RBC ($p < .01$), and HCT ($p < .01$) at the highest tested dose (7.44 mg Co/kg bw/day as $CoCl_2$). The female animals' PLT and Reti counts were not affected.

Histopathology revealed a significant and dose dependent increase in erythroid hyperplasia in the bone marrow of male and female animals treated with 2.48 mg (4/10 males and 7/10 females) and 7.44 mg Co/kg bw/day (7/10 animals for both sexes) compared with the controls and the animals treated with 0.74 mg Co/kg bw/day (0/10 animals with histopathological or microscopic changes in the bone marrow in these groups). All changes in hematology and bone marrow observed at the end of the treatment period (days 91/92) were reversible following a 4-week recovery period. Hematological parameters were compared between the highest dose group and control at day 119, showing that there were no differences between the groups. A comparison of the high-dose group's blood parameters at days 91/92 and day 119 showed that any effect at cessation of treatment had subsided at the end of the recovery period (Table 1, recovery groups).

Under the conditions of this study, $CoCl_2$ had a significant effect on hematological parameters from a dose level of 2.48 mg Co/kg bw/day (males) and 7.44 mg/kg bw/day (females). Male or female reproductive parameters were not affected in this study at any dose level (Table 6).

Tricobalt tetraoxide: Subchronic RDT study with reproductive toxicity screening (OECD 408). None of the animals died prematurely. No test item-related changes were noted for neurological screening, behavior, external appearance, body posture, or movement and coordination capabilities, food and drinking water consumption, feces, estrous cycle urinalysis, hormone levels, ophthalmoscopic examination, macroscopic postmortem findings, or organs weights including testes and prostate. There were no macroscopic or histopathological findings in heart or thyroid at any dose. A dark staining of the feces was noted for all test item-treated animals. The intensity of the staining increased with the dose level. This finding is considered to be related to the black-gray color of the test item and not a toxicological effect, due to the absence of any gastrointestinal findings (no irritation nor bleeding).

At the high dose of 734 mg Co/kg bw/day as Co_3O_4, a slightly reduced body weight, body weight gain, and body weight at autopsy were observed in the males. The female animals were only marginally affected.

Starting at the intermediate dose of 220 mg Co/kg bw/day (as Co_3O_4), increased values were noted for HGB, number of erythrocytes, and HCT. These changes were statistically significant at $p < .01$, however, all changes remained below a 10% difference from the control values. In the females, only the HGB level was affected ($p < .01$). In contrary to the $CoCl_2$ exposed animals, exposure to Co_3O_4 did not result in any changes in Reti or PLT count, nor were there any changes in the bone marrow of the Co_3O_4-treated animals, even at the highest dose.

At the dose of 1000 mg substance (= 734 mg Co/kg bw/day), the hematological effects consistently exceeded a 10% difference from the controls, occurred in males as well as in females with statistical significance ($p < .001$) (Table 2A and B). The highest dose level was considered to be the lowest observed adverse effect level (LOAEL) for the Co-related hematological effect, even in the absence of histopathological findings in the bone marrow.

No test item-related effects were observed on any of the remaining measured endpoints (data not shown).

At the end of the 4-week recovery period (restricted to the control and high-dose group), the color of feces had returned to normal, and body weight as well as hematological parameters of the high-dose animals were within the range of the control group, indicating a complete recovery (Table 2, recovery groups). Also the comparison of the affected endpoints in the high-dose animals at days 91/92 versus day 119 showed that the treatment-related effects were reversible.

Under the conditions of this study, Co_3O_4 had a significant effect on hematological parameters at the limit dose of 734 mg Co/kg bw/day. The intermediate dose was considered to be the no observed adverse effect level (NOAEL) due to the lack of effect on the bone marrow and on body weights. Furthermore, the hematological findings were less pronounced (fewer

parameters affected, sex-specific, small magnitude of change) and were reversible at least at the highest dose. Male or female reproductive parameters were not affected in this study at any dose level (Table 6).

In addition to the determination of NO(A)ELs and LO(A)ELs based on presence or absence of significant effects, BMD modeling was performed on all $CoCl_2$ and Co_3O_4 hematological dose response data (32 datasets). Importantly, the absence or presence of the histopathological correlate of the hematology results (bone marrow hyperplasia) has not been considered in the BMD analysis, as these data represent semiquantitative histopathology scores. These data are, however, of high biological relevance on whether to judge a result as "adverse" or "significant" and are taken into account when evaluating the overall data.

Most of the BMD models did not provide an acceptable fit to most of the datasets using either a constant or a nonconstant variance model and with normal distribution. Only exponential models provided adequate goodness-of-fit p value for 5 of the 32 datasets. For example, using a constant variance model, the male RBC dataset from oral exposure to $CoCl_2$ was fitted by an Exponential 5 model resulting in a BMD1STD and BMDL1STD of 2.40 and 1.20 mg Co/kg bw/day, respectively, indicating "no effect" at 1.2 mg Co/kg bw/day and a 10% response dose at 2.4 mg/kg bw/day, matching the NOAEL/LOAEL determination. For female rats, an adequate fit was provided by an Exponential 2 model for the PLT dataset resulting in a BMD1STD and BMDL1STD of 14.0 and 6.2 mg Co/kg bw/day, respectively. However, the ANOVA for this dataset resulted in p value of .28 and the data were not taken forward to a groupwise comparison for significant differences. Adequate fit was also provided by an Exponential 4 model for male HCT dataset using a nonconstant variance model resulting in a BMD1STD and BMDL1STD of 0.79 and 0.44 mg Co/kg bw/day, respectively. These values are somewhat below the NOAEL/LOAEL bracket (0.74/2.48 mg Co/kg bw/day).

For the Co_3O_4 datasets, in male rats, only the PLT dataset was adequately fitted by Exponential 2 model with nonconstant variance resulting in a BMD1STD and BMDL1STD of 317 and 219 mg Co/kg bw/day, respectively. For female rats, only an Exponential 5 model with nonconstant variance provided adequate fit for the HGB dataset resulting in a BMD1STD and BMDL1STD of 210 and 115 mg Co/kg bw/day, respectively.

Tricobalt tetraoxide: Combined RDT study with the reproduction/developmental toxicity screening test (OECD 422). None of the animals died prematurely during the course of the study.

No test item-related influence was noted in neurological observational or functional screening of male or female rats treated with 73.4, 220, and 734 mg Co (as Co_3O_4)/kg bw/day. No test item-related influence was observed on drinking water consumption of the male and female animals and no influence was noted on the body weight during the entire study. The food intake of the intermediate dose dams (220 mg Co/kg bw/day) was reduced on lactation day 1 by 30% and statistically significantly reduced by 49% at the high dose ($p \leq .01$) compared with the control. On day 4 of the lactation period the food consumption of the females of the intermediate and high-dose groups were elevated due to a compensatory effect but still reduced by 16% or 15%, respectively, compared with the control group. No test item-related changes in hematological or biochemical parameters were noted at the end of the premating period (test day 15) of male and female rats. Macroscopic inspection at necropsy revealed no test item-related changes in the organs or tissues of male or female rats after treatment with either 73.4, 220, or 734 mg Co (as Co_3O_4)/kg bw/day. There was no test item-related influence noted on organ weights or histomorphological examination of organs.

With respect to fertility and reproduction parameters, no test item-related influence was noted on the fertility of the female rats, precoital time, gestation length, or on sperm number, viability and morphology in the male rats. There were no test item-related differences in the number of corpora lutea, implantation sites, in the number and sex of pups, runts or malformed pups, in the values calculated for the gestation length, the birth index and the live birth index between the control group and the animals treated with 73.4, 220, and 734 mg Co (as Co_3O_4)/kg bw/day. There were no test item-related increases in preimplantation loss or postimplantation loss noted in the dams after treatment at any dose.

No test item-related deaths occurred in the F1 generation (pups) at the low and intermediate dose. At the high dose, the viability index of the pups (87.6%) was statistically significantly decreased at $p \leq .01$ compared with the control group (100%). This finding was due to the total loss of 1 litter. Pups otherwise showed no abnormal behavior, no test item-related influence on the total litter weight at any of the tested dose levels, and no external abnormalities. There was no test item-related influence noted on the mean and total litter weight at 73.4 or 220 mg Co (as Co_3O_4)/kg bw/day. The mean body weight of male, female, and total pups was reduced in the high-dose level group administered to the parental F0 generation, being statistically significant (at $p \leq .01$) on lactation day 0/1 (up to 18% below the control) and lactation day 4 (up to 21% below the control). There was however, no change in the total litter weight of the pups (sum of weights of all pups from 1 litter). Examinations at dissection revealed no external abnormalities in any of the pups examined.

Under the conditions of this study, Co_3O_4 had no effect on any parameter measured in the parental animals (F0). There were no abnormalities or variations in any of the pups. Tricobalt tetraoxide was considered to be nontoxic to the F0 generation in this study. Male or female reproductive parameters were not affected in this study at any dose level. There was a significant reduction in mean body weight of the pups at the highest dose tested (734 mg Co/kg bw/day).

Cobalt sulfide: Combined RDT study with the reproduction/developmental toxicity screening test (OECD 422). This study followed the same study design and same level of detailed examination as the above OECD 422 study with Co_3O_4. There was a complete absence of findings. The results of this study are summarized in Table 7 and reported below in an abbreviated form.

None of the exposed animals died prematurely during the course of the study.

There was no test item-related influence on any of the parameters measured in this study in both the F0 and the F1 generation. Based on this study, CoS was considered to be nontoxic up to the limit dose to the F0 and F1 generation.

DISCUSSION

The hypothesis that differences in *in vivo* effects between "inorganic cobalt substances" are related to differences in their solubility in biologically relevant fluids, with less soluble cobalt salts being less likely to cause toxicity, is well supported by the tiered data presented.

Table 7. Combined RDT Study With Reproduction/Developmental Toxicity Screening Test With Cobalt Sulfide and Tricobalt Tetraoxide (OECD 422

	Cobalt Sulfide	Tricobalt Tetraoxide
Doses (as cobalt)	65, 194, 648 mg Co/kg bw	73.4, 220, 734 mg Co/kg bw
NOAEL (F0 generation)	648 mg Co/kg bw/day Based on nonadverse piloerection in a few male and females at 100 mg/kg/day and higher	734 mg Co/kg bw/day Based on nonadverse findings of piloerection and decrease food consumption during lactation
NOAEL (reproductive toxicity)	> 648 mg Co/kg bw/day No test item-related influence was noted on mating behavior, fertility, implantation, the gestation length, or the birth index at any dose tested	> 734 mg test item/kg bw/day No test item-related influence was noted on mating behavior, fertility, implantation, the gestation length, or the birth index at any dose tested
NOAEL (F1 generation)	> 648 mg Co/kg bw/day No test item-related influence was noted on the growth and development of the offspring from conception until sacrifice on day 4 postpartum or shortly thereafter.	220 mg Co/kg bw/day Based on the significant reduction in mean body weight of pups in 1 dam at 734 mg Co/kg bw/day

NOAELs for F0 generation and reproductive toxicity.

The public domain contains reports of several cobalt-related effects at high doses. The reported target organs are the testes, thyroid, and the heart. Adverse cardiac effects were observed in male SD rats following 3-month exposure to $CoCl_2$ at doses of 32 mg Co/kg bw/day (Domingo *et al.*, 1984). Histopathological changes in the thyroid were reported in female mice following 15-day exposure to cobalt as $CoCl_2$ at doses of approximately 50 mg Co/kg bw/day (reviewed and dose calculated by Finley *et al.* (2012), based on Shrivastava *et al.*, 1996). Adverse effects on testes, such as atrophy, congestion, or infarction, were observed following 10-week exposure to $CoCl_2$ at a dose of 20 mg Co/kg bw/day (Nation *et al.*, 1983). These studies did not include a thorough reporting on animal morbidity, body weights, or food and drinking water consumption, nor were robust dose response data generated. In the studies presented here, male rats experienced a statistically significant body weight reduction of 11% (following 90-day exposure to $CoCl_2$ at 7.44 mg Co/kg bw/day), and changes in hematological parameters of up to 30% compared with control. In this study design, no effects on testes, thyroid, or heart at the level of organ weights, gross pathology, histopathology, and standard clinical biochemistry were observed. Although it cannot be ruled out that the male reproductive, thyroid, or heart effects may have occurred at higher doses, these doses have led to mortality in range-finding studies associated with our work program. Dosing to excessive morbidity or mortality must be avoided and cannot be included in regulatory studies, which have to follow OECD guidelines. The maximum tolerated dose (MTD) is defined by the OECD as the dose inducing slight toxic effects (for example, abnormal behavior or reactions, minor body weight depression, or hematopoietic system cytotoxicity). Dosing to weight reductions beyond 10% and hematological changes greater than 30%, or to any level of severe morbidity cannot be reconciled with the OECD principle of MTD and was avoided in the present studies.

In the absence of effects on reproductive parameters, effects levels, and dose response relationships were determined based on the observed hematological and bone marrow effects. The analysis by ANOVA followed by post hoc *t* test gave a good resolution of magnitude of effect between the doses and control, yielding NOELs at the lowest dose consistently and LO(A)ELs at the mid dose in the studies with $CoCl_2$ and Co_3O_4. Clear effects, exceeding a 20% difference from control at least in the males, were seen at the highest dose. The mid dose of the Co_3O_4 dataset yielded statistically significant results in 2 of 4 affected endpoints in males, and in 1 of 3 affected endpoints in females. These changes remained below a 10% difference. Also, there was an absence of the effect on PLT and Reti, as well as the absence of the bone marrow findings at all Co_3O_4 doses, whereas these changes were present in the $CoCl_2$ exposed animals already from the mid dose. These considerations, as well as nonaffected body weights, are reflected in the determination that the Co_3O_4 mid dose represents a LOEL, but not a LOAEL.

The use of the BMD approach was complicated by the large number of datasets that were not amenable to modeling, primarily due to large differences in variance among doses within each experiment. However, the 5 BMDs and corresponding BMDLs were not inconsistent with judgments of NOAELs and/or LO(A)ELs, lending support to the use of the NOAELs/LOAELs, in combination with histopathological- and other data, as a suitable description of the dose responses.

We found a large variation in the biological behavior of Co compounds, from very reactive to relatively inert, despite the fact that all Co compounds appear to be excreted within 72-h postoral application. More than 99% of the water-insoluble Co_3O_4 is excreted via the feces, suggesting that Co_3O_4 is only absorbed biologically to a small extent, and becomes bioavailable systemically only to a very small fraction of its external dose (Tables 4 and 5). This observation is matched by the very low toxicity of Co_3O_4 after both acute and repeat dose toxicity studies (Tables 3, 6, and 7). In contrast, more than 80% of the water-soluble $CoCl_2$ is excreted via the feces, indicating that up to 12% of $CoCl_2$ becomes systemically available at least in male rats (also Tables 4 and 5). These data are in good agreement with the literature (Kirchgessner *et al.*, 1994) and are matched by the higher toxicity of $CoCl_2$ after both acute and repeat dose toxicity studies (also Tables 3, 6, and 7), while also being excreted via urine within 72 h as shown by 100% mass balance within 72 h (Table 5).

At the high bolus doses applied in this study design, only a maximum of 12% of $CoCl_2$ was bioavailable. This is lower than what would be predicted based on studies in humans (Tvermoes *et al.*, 2014) or on Co-specific biokinetic models (Unice *et al.*, 2012, 2014). In human volunteer studies, the absorption of Co from $CoCl_2$ was estimated to be 20% in men and 45% in women (Tvermoes *et al.*, 2014). The difference between the present study and the published data may be related to the differences in study design: the dose in the studies with humans by Tvermoes and Unice was much lower with 0.08–0.19 mg Co/kg bw/day, as

opposed to > 2 mg Co/kg bw/day in the rodent study presented here. Furthermore, Tvermoes *et al.* exposed the human volunteers for a 3-month period and the biokinetic model by Unice *et al.* is assuming at least 10 days of Co administration, whereas the present TK study employed a single bolus dose design, followed by a 72 our follow up. Clearly, the studies in humans and the biokinetic model provide a more realistic and relevant estimate of the bioavailability of Co from $CoCl_2$ than does the present plasma TK study in rodents. The aim of the study presented here was the comparative estimation of *in vivo* bioavailability between different substances of interest, so that the bioavailability of Co from lesser-studied Co compounds can be put into context with the bioavailability of $CoCl_2$, which is a data-rich substance. The higher uptakes of Co at lower chronic doses versus a high bolus dose can be reconciled with the potential saturation of transport mechanisms at acute high exposures. The higher estimated Co absorption in women (Tvermoes *et al.*, 2014) is also reflected in the somewhat higher bioavailability in female versus male rats observed in the rat TK study. This is a potential result of the generally greater iron demand in females of reproductive age, and supports the evidence for shared intestinal absorption mechanism, used for both Co and Fe uptake.

The evidence presented by Tvermoes and Unice in humans, together with the rat data shown here, provide no indication that there would be bioaccumulation of the Co ion. This observation is also supported by the complete reversibility of the effects observed at the end of a 90-day treatment period in the present RDT studies following a 30-day washout period.

Following absorption in the gastro intestinal tract, tissue level increases appear to reflect passive distribution of Co to most organs. The elevated uptake (approximately 100-fold increase in Co concentration compared with untreated controls) of Co into the bone marrow correlates with the nature of the most sensitive endpoint of its toxicity, namely increases in local erythrocyte production (erythroid hyperplasia in the bone marrow). This is evidenced also by hematological changes and an increased oxygen carrying capacity of the blood, and also correlates with the essentiality of Co in vitamin B12 for hematopoiesis. It cannot, at this stage, be explained why Co_3O_4 caused hematological changes in the absence of erythroid hyperplasia of the bone marrow, as was observed with $CoCl_2$. Further studies would be required to investigate, for example, the tissue distribution of cobalt following Co_3O_4 intake.

The very low bioavailability of Co_3O_4 necessitates the application of extremely high doses of it, and similar substances, to obtain analytically detectable concentrations of cobalt in the exposed biological matrix. In Supplementary Table 2, comparable urinary Co concentrations were achieved following $CoCl_2$ and Co_3O_4 PO exposure. Tricobalt tetraoxide, however, had to be applied at a dose that was 100 times higher in Co equivalent compared with $CoCl_2$, to generate urinary concentrations suitable for a parallel Co analysis. This shows that the mere presence of Co in a substance does not necessarily confer the expected "dose to target" or effect, and that consideration of the differences among the members of the Co category is fundamental to these compounds' assessment.

The introduction of *in vitro* bioaccessibility allowed us to test the read-across hypothesis that solubility in artificial biological media correlates with systemic toxicity. The information gained in a number of toxicological tests and toxicokinetic investigations verified this initial hypothesis for a number of representative cobalt substances. It is important to mention that the representatives included in the presented read across all have counter-ions with no or negligible toxicity. This is why the Co ion is considered the "toxic unit" without a significant contribution of the counter-ion.

Results for bioelution for the range of the selected cobalt compounds extend over 3 orders of magnitude, whereas results for water solubility extend over 6 orders of magnitude. This difference in range between water solubility and bioelution underlines the potential value of bioelution testing, as it is likely more representative of the *in vivo* bioavailability and toxicity of the Co substances. In fact, the bioelution results follow the pattern established in the acute and RDT studies in that low bioelution results are associated with lower toxicity and higher bioelution results are associated with higher toxicity of these selected Co compounds (Tables 3, 6, and 7).

Similar tiered approaches have previously been used as read-across categories for oral toxicity by other metal compound families including nickel (Henderson *et al.*, 2012) and indium (Lombaert *et al.*, 2018). For example, in their evaluation of 12 nickel compounds, Henderson *et al.* (2012) compared oral LD50 values with gastric and intestinal nickel ion release determined by bioelution and identified that the percent of available nickel released after 2 h in synthetic gastric fluid provided the strongest relationship. Compounds with less than 48% nickel ion release after 2-h bioelution in simulated gastric fluid were predicted to exceed an acute oral LD50 of 2000 mg/kg bw with 95% confidence.

Our results are also consistent with the fact that gastric-insoluble Co compounds are not classified for oral toxicity based on the premise that the primary toxic moiety of metal compounds is the metal ion, and therefore the more soluble the compound, the greater the potential for oral toxicity (Henderson *et al.*, 2012).

In summary, these studies, when considered in a weight of evidence approach, show that *in vitro* solubility, and specifically bioelution in simulated gastric fluid, is a good predictor of *in vivo* bioavailability and oral toxicity of inorganic Co compounds. Bioelution therefore appears to be a valuable tool in the hazard analysis toolkit for the grouping and read across of Co substances' classifications and risk assessment parameters. Furthermore, a tiered approach to studying these compounds is proposed that reduces experimental animal testing, expedites evaluations, and, importantly, maintains confidence in judgments of potential health risk from this diverse set of technologically useful compounds.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper beyond employment by the funding organizations (RD, VV) or by a member company of such organizations (SV), having received funding for help in preparing this manuscript (MD, LW), or having previously consulted CoRC (AB).

FUNDING

This work was funded by the organizations Cobalt Institute and Cobalt REACH Consortium (CI/CoRC).

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