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Bioaccessibility Testing of Cobalt Compounds

Woodhall Stopford, MD, MSPH; John Turner, PhD; Danielle Cappellini, BSc, MHA; Tom Brock, PhD, DABT

Abstract

Testing of metal compounds for solubility in artificial fluids has been used for many years to assist determining human health risk from exposure to specific compounds of concern. In-lieu of obtaining bioavailability data from samples of urine, blood, or other tissues, these studies measured solubility of compounds in various artificial fluids as a surrogate for bioavailability. In this context, the measurement of metal "bioaccessibility" can be used as an *in vitro* substitute for measuring metal bioavailability. Bioaccessibility can be defined as the availability of metal for absorption when dissolved in body fluids or juices, or their *in vitro* surrogates.

The aim of this study was to measure and compare the bioaccessibility of selected cobalt compounds in artificial human tissue fluids and human serum. A second aim was to initiate studies to experimentally validate an *in vitro* methodology that would provide a conservative estimate of cobalt bioavailability in the assessment of dose from human exposure to various species of cobalt compounds.

This study evaluated the bioaccessibility of cobalt (II) from 11 selected cobalt compounds and an alloy in 2 physical forms in 5 surrogate human tissue fluids and human serum. Four (4) separate extraction times were used up to 72 hours. The effect of variables such as pH, dissolution time, and mass-ion effect on cobalt bioaccessibility were assessed as well. We found that the species of cobalt compound as well as the physico-chemical properties of the surrogate fluids, especially pH, had a major impact on cobalt solubility. Cobalt salts such as cobalt(II) sulfate heptahydrate were highly extractable, whereas cobalt alloys used in medical implants and cobalt spinels used as pigments, showed minimal dissolution over the period of the assay.

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Bioaccessibility Testing of Cobalt Compounds

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Introduction:

Testing of metal compounds for solubility in artificial fluids has been used since the turn of the last century for determining health risk from exposure to specific compounds of concern. As opposed to obtaining blood or organ values for the compound of interest (e.g. measuring bioavailability), these studies measured compound solubility in various artificial fluids as a surrogate for bioavailability testing. Within this context, the measurement of metal "bioaccessibility" can be used as an *in vitro* substitute for measuring metal bioavailability. Bioaccessibility is defined as the availability of a metal for absorption when dissolved in body fluids or juices or their *in vitro* surrogates [38].

The aims of this study were two-fold: The first was to measure and compare the bioaccessibility of selected cobalt (II) compounds in surrogate human tissue fluids and human serum. A second purpose was to initiate studies to experimentally validate an *in vitro* methodology that would provide a conservative estimate of cobalt bioavailability in the assessment of dose from exposure to various species of cobalt compounds. Although early studies evaluated risks associated with ingestion and, consequently used a weak acid leaching method as a measure of bioaccessibility, similar extraction studies can be used to judge bioaccessibility of cobalt at other sites, including the skin, lung, joints and blood. These *in vitro* studies reviewed are summarized below.

One bioaccessibility study indicated that cobalt cations are primarily associated with low molecular weight compounds that would be diffusible and highly mobile. This study Weinzierl & Webb[1] examined the dissolution of metallic cobalt in horse serum and homogenates of rat tissues, and binding to proteins and small, diffusible molecules. They found that cobalt cations were bound primarily to small, diffusible molecules and not to proteins, as indicated by cation analysis before and after dialysis.

Edel, et al.[2], conducted leaching experiments with neutron-irradiated metallic cobalt dust. They found that cobalt was highly soluble in lung cytosol and plasma, compared to other metals (tantalum and tungsten). Test fluids were human venous blood samples and

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human lung tissue homogenized with sucrose and ammonium acetate. In gel filtration experiments with plasma and lung cytosol fluid, cobalt was bound to three biochemical pools. Most of the element (56%) was associated with low molecular weight compounds that would be diffusible and highly mobile. Another fraction (34%) was associated with proteins of a size (MW 70,000-80,000 Daltons) that could include transferrin. Only 8% of the cobalt was associated with high molecular weight components.

Another study Collier, et al.[12], varied concentrations of citrate and bicarbonate to simulate the pH of extracellular fluid, cytoplasm and alveolar macrophage lysosomes. At the pH of lysosomes (4.5) cobalt oxide dissolved at significantly higher rates than at the pH of cytoplasm (6.1) or extracellular fluid (7.2).

An *in vitro* study Takahashi and Koshi[4] examined cobalt solubility in distilled water, fetal calf serum, 2% albumin, 2% glycine, and 0.1% disodium ethylenediaminetetraacetate (NaEDTA) solution. Metallic cobalt, cobalt sulfide, and cobalt oxides (CoO, Co_2O_3) were suspended in the solutions and supernatants were analyzed for cobalt. Metallic cobalt had the greatest dissolution in 0.1% NaEDTA (15.1%), followed by 2% glycine, fetal calf serum, 2% albumin, and water (0.4%). The cobalt compound cobalt sulfide dissolved most completely in 2% glycine (48.2%) but significant dissolution occurred in 0.1% NaEDTA, fetal calf serum, and 2% albumin. In contrast, the cobalt oxides showed minimal solubility in these solutions; cobalt from Co_2O_3 was most soluble in 0.1% NaEDTA (4.9%) and fetal calf serum (1.6%).

Methodology

We evaluated the bioaccessibility of 11 cobalt compounds and an alloy in two physical forms and compared the results to the bioaccessibility of cobalt sulfate heptahydrate. The following materials were provided by Cobalt Development Institute from their test-sample repository, Particle Technology Laboratories, Inc., (Downers Grove, IL) for testing:

Compound	% Cobalt
Cobalt Sulfate Heptahydrate (CASRN 10026-24-1)	21.0%
Cobalt Neodecanoate (CASRN 27253-31-2)	21.0%
Cobalt Monoxide (CASRN 1307-96-6)	78.0%
Cobalt Chloride (CASRN 1332-82-7))	25.0%
Cobalt Carbonate (CASRN 7542-09-8)	45.0%
Cobalt Aluminate Spinel Pigment (CASRN 68186-86-7)	23.6%

Compound	%Cobalt
Cobalt Tungsten Carbide (Pre-Sintered; CASRN 12070-12-1)	30.0%
Cobalt Tungsten Carbide (Post-Sintered; CASRN 12070-12-1)	30.0%
Cobalt Naphthenate (CASRN 61789-51-3)	10.0%
Cobalt Octoate (CASRN 136-52-7)	15.1%
Cobalt Extra Fine Powder (CASRN 7440-48-4)	99.9%
Stellite 21 Powder - Metallic Alloy of Cobalt Chrome (Batch A004365)	59.7%
Stellite Rods - Metallic Alloy of Cobalt Chrome (Batch Z012559, 4 mm diameter)	59.7%

A. Materials and Equipment

Mettler AE 200 Electronic Analytical Balance (Readability 0.1mg)

Pyrex Brand Low Actinic Erlenmeyer Flasks - 250 ml (Corning # 55020-250)

Pyrex Brand Erlenmeyer Flasks – 125 ml (Corning # 4980-125)

Fisher Scientific Accumet 50 pH Meter with Orion Model #8104 Ross Combination pH electrode

Precision Reciprocal Shaking Bath Model 50 (37 +/- 1□C; 150 oscillations/min.; stroke length = 1 inch)

0.45 μm Membrane Filters - 142 mm (Fisher Scientific #09-719-2F)

142mm Hazardous Waste Filtration System (Millipore #YT30-142-HW)

5% Carbon Dioxide in Nitrogen Compressed Gas (MG Industries)

Lab Crest, Mark III Flowmeter with Tube #448-118 and Stainless Steel Ball (Setting 2 = approx. 50 cc/min)

Fisher Scientific Vortex Genie 2 Vortex Mixer

50 ml Polypropylene Centrifuge Tubes with Screw Caps (Milian # CT-50NS)

IEC Model K Centrifuge

Varian SpectrAA 600 Flame Atomic Absorption Spectrophotometer with Deuterium Background Correction

Perkin Elmer 5100 Flame Atomic Absorption Spectrophotometer with Deuterium Background Correction

Perkin Elmer 4100

ZL Furnace Atomic Absorption Spectrophotometer with AS70 Autosampler

Cobalt Hollow Cathode Lamps (Perkin Elmer # 303-6021 and Varian #5610123200)

B. Extraction Methods

Five surrogate tissue fluids were used in the study: interstitial fluid, lysosomal (intracellular) fluid, gastric juice, intestinal juice, and alveolar fluid. Artificial synovial fluid was used for the extraction of the alloy only. Human serum was also used as an extraction medium.

In order to get sufficient data to calculate dissolution halftimes, we measured cobalt bioaccessibility after four different extraction periods (2, 5, 24, and 72 hours). Johanssen, et al.[5], were able to estimate dissolution halftime of 11 years using a three day extraction time. The extraction experiments were prepared by mixing 1.0 gram of compound/alloy with 50 ml of surrogate fluid (when using serum, 0.5 gram of compound was mixed with 25 ml of serum for economic reasons). Each mixture was placed in a reciprocating shaker bath set to shake at 150 oscillations/min (stroke length = 1 inch) at a temperature of $37° \pm 1^{o}$ C for the specified extraction period. The mixture was then filtered through a 0.45 um filter to remove any solids. The filtrate was analyzed for cobalt by flame absorption spectrophotometry.

All extractions were prepared and analyzed in duplicate. If the filtrate for any of the extractions performed for two hours showed greater than 250 ppm cobalt, another extraction was performed using 100 mg of test compound/alloy per 50 ml of test fluid. This was done to avoid erroneous low values caused by mass ion effect.[3] Also, once 50% of a test compound/alloy had been solubilized, no additional extractions were carried out on that compound/alloy.

A method detection limit (MDL) was determined for cobalt. Seven standards were prepared at a concentration of 0.05 mg/l cobalt and analyzed by flame atomic absorption spectroscopy. A standard deviation (S) was calculated. The method detection limit was then determined by multiplying the standard deviation (S) by the students' T value corresponding to the number of standards analyzed (for 7 standards the students' T value is 3.143).

Also, a percent recovery was determined for cobalt in each extraction fluid. A soluble cobalt standard was used to spike the extraction fluids at a concentration of 10 mg/l.

Each spiked fluid was taken through the entire extraction procedure one time using the two hour extraction period. Each filtrate was then analyzed for cobalt and a percent recovery was calculated.

1. Artificial Gastric Juice and Intracellular Fluid

The compounds were weighed in duplicate into 250 ml amber Erlenmeyer flasks (approx. 1.0 g for the straight extractions and 0.1 g for the 1:10 dilutions). Fifty (50) milliliters of fluid were added. The flasks were swirled to mix the compounds and the fluid. The pH of the solution was checked to insure that it was at the required level (1.5 for gastric juice and 4.5-5.0 for intracellular fluid). 2N HCl or 1N NaOH was used for adjustment. The openings of the flasks were covered with parafilm and aluminum foil and placed into a preheated 37°C reciprocal shaker bath. After the appropriate extraction time (2, 5, 24, and 72 hours), the solutions were removed from the bath. The pH was checked and the solutions were filtered through a 0.45 um filter using the Millipore filtration apparatus. The filtrates were collected in 8 oz. disposable plastic bottles and kept in a 35°C incubator until analysis.

2. Artificial Intestinal, Interstitial, and Alveolar Fluids

The compounds were weighed in duplicate into 250 ml amber Erlenmeyer flasks (approx. 1.0 g for straight extractions and 0.1 g for the 1:10 dilutions). Fifty (50) milliliters of fluid were added. The flasks were swirled to mix the compounds and fluid. The pH of the solution was checked to insure that it was at the required level of 7.4. 2N HCl or 1N NaOH was used for adjustment. For all compounds except cobalt sulfate, the pH of the extractions would drift upward over time. It was necessary to use 5% CO_2 in nitrogen to maintain a pH of 7.4 throughout the extraction. In this case, the flasks were fitted with a No.6 2-hole rubber stopper with glass tubing inserted in each hole. One piece of the tubing extended below the surface of the fluid and the other piece served as a means for ventilation of the flask. The CO_2 was bubbled into the solution a rate of approximately 50 cc/min. The bubbling solutions were placed in a preheated 37°C reciprocal shaker bath. After the appropriate extraction time (2, 5, 24, and 72 hours), the solutions were removed from the bath. The pH was checked and the solutions were filtered through a 0.45 um filter using the Millipore filtration apparatus. The filtrates were collected in 8 oz. disposable plastic bottles and kept in a 35°C incubator until analysis.

3. *Artificial Synovial Fluid*

This fluid was only used with the Stellite alloy samples (rod and powder). The samples were weighed in duplicate (0.5 g) into 125 ml Erlenmeyer flasks and 25 ml of fluid were added. The pH was checked to insure that it was 7.4. In all cases, no pH adjustment was necessary. The openings of the flasks were covered with Parafilm and then the entire flask was wrapped with aluminum foil. The flasks were placed in a preheated 37°C reciprocal shaker bath. After the appropriate extraction time (2, 5, 24, and 72 hours), the solutions were removed from the bath and the pH was checked. The solutions were decanted into 50 ml screw cap centrifuge tubes and stored at 4^{o} C until analysis.

4. *Serum*

The compounds were weighed in duplicate into 50 ml screw cap centrifuge tubes (approx. 0.2 g for straight extractions and 0.02 g for the 1:10 dilutions). Ten (10) milliliters of serum were added. The solutions were mixed with a vortex genie for 30 seconds. The tubes were placed in a centrifuge tube rack that was mounted in the preheated 37°C reciprocal shaker bath. After the appropriate extraction time (2, 5, 24, and 72 hours), the solutions were removed from the bath. The tubes were centrifuged for 10 min. to separate the compounds from the serum. The serum layer was removed with a disposable Pasteur pipette and collected in a 15 ml screw cap centrifuge tube. The samples were stored in the refrigerator until analysis.

C. Extraction Fluids:

A. Artificial Interstitial Fluid

Gamble's solution has been used for more than 30 years for determining the solubility of nucleotides and manmade fibers. It represents the interstitial fluid deep within the lung. Citrate is used in place of protein and acetate to represent organic acids. Either the reaction vessels have air-tight caps or 5% CO_2 is bubbled through the solution maintain the pH at 7.4.

The make up of the solution is follows (in g/L of DI water)[6]:

Magnesium chloride hexahydrate	0.2033
Sodium chloride	6.0193
Potassium chloride	0.2982
Dibasic sodium phosphate (anhydrous)	0.1420
Sodium sulfate (anhydrous)	0.0710
Calcium chloride dihydrate	0.3676
Sodium acetate trihydrate	0.9526
Sodium bicarbonate	2.6043
Sodium citrate dihydrate	0.0970

An interlaboratory study has been conducted looking at long-term durability of manmade fibers using Gamble's solution as the test medium.[7] Experiments comparing the solubility of manmade fibers in rodent lungs compared with this artificial interstitial fluid show similar results.[8] Gamble's solution has been used to investigate the solubility of various metals, including uranium hexafluoride, indium phosphide, and cobalt.[5,9,10]

B. Artificial Lysosomal Fluid

An analogous fluid to artificial extracellular fluid has been developed for investigating the long-term durability of man-made fibers after phagocytosis into cells. In addition to differences in salt composition, the phagolysosomal pH of alveolar and interstitial macrophages has a pH of 4.5-5.[11-12,39] The acidity of this intracellular fluid may explain the higher level of solubility of metals that are phagocytized versus those that remain extracellular.[13] The composition of this fluid is as follows[14] (expressed in g/L of DI water):

NaCl	3.210
NaOH	6.000
Citric acid	20.800
CaCl	0.097
NaHPO4.7H20	0.179
Na_2SO4	0.039
MgCl.6H20	0.106
Glycerine	0.059
NaCitrate.2H20	0.077
NaTartrate.2H20	0.090
NaLactate	0.085
NaPyruvate	0.086
Formaldehyde	1.000 (ml/L)

Similar man-made fibers have been tested for durability in this fluid[14] and in rodent lungs[8]. However, interlaboratory testing of this procedure has not been conducted.

C. Artificial Gastric Juice

Surrogates of gastric juice have been used for testing metal solubility for over a century. In 1982 The Comite Europeen de Normalisation developed EN71.3, Safety of Toys: Migration of Certain Elements. This standard method evaluates solubility of metals in 0.07N hydrochloric acid at 37^{o} C maintained at a pH of 1.5. This method has been adopted in the United States as ASTM D5517: A Standard Method for Determining the Solubility of Metals in Art Materials. The method has undergone extensive interlaboratory round robin testing. There is a large database for various cobalt compounds against this test (Stopford, unpublished data).

This method has been compared with in vivo solubility of lead silicates in the stomach of rodents.[15.16] When compared to synthetic gastric juice containing organic acids and enzymes, ASTM D5517 shows equivalent results.

D. Artificial Intestinal Juice

Artificial intestinal juice mirrors the composition of human small intestinal juice after a meal. It was initially developed to investigate the availability of dyes for absorption or azoreduction (Stopford, unpublished data). This fluid has been investigated for the solubilization of lead ions from lead silicates and compared to solubilization in the small

intestinal tract of rodents.[15.16] The composition of this fluid is as follows (in g/L of DI water):

NaCl	6.79
KCl	0.52
NaHCO3	1.85
CaCl2	0.11
Sodium taurocolate	1.07

The pH is adjusted to 7.4.

E. Artificial Alveolar Fluid

Gamble's solution represents the basal interstitial fluid deep within the lung, and has been used as a lung fluid simulant [9]. However, alveolar fluid (AF) also contains phospholipids with surfactant properties that prevent alveolar collapse at low lung volumes [17]. A primary phospholipid identified in human bronchoalveolar lavage (BAL) fluid is phosphatidylcholine – 65% of total phospholipid. In bronchoalveolar lavage fluid of humans, the median concentration was found to be 0.0074 g of phosphatidylcholine/L of BAL fluid. The dilution factor for BAL fluid has been measured in lambs comparing urea in BAL fluid to urea in plasma.[12] BAL fluid contained 1/13.5 of this constituent when compared to plasma. Consequently, a synthetic alveolar fluid could include phosphatidylcholine (in g/L of DI water):
:

Magnesium chloride hexahydrate	0.2033
Sodium chloride	6.0193
Potassium chloride	0.2982
Dibasic sodium phosphate (anhydrous)	0.1420
Sodium sulfate (anhydrous)	0.0710
Calcium chloride dihydrate	0.3676
Sodium acetate trihydrate	0.9526
Sodium bicarbonate	2.6043
Sodium citrate dihydrate	0.0970
Phosphatidyl choline	0.1000

F. Artificial Synovial Fluid

Artificial synovial fluid has been used for the investigation of metal binding properties but has not undergone the more rigorous comparative investigations undertaken for artificial gastric juice or artificial interstitial fluid. The composition of this fluid is as follows[18]:

Tris-NaCl 0.05 M(Sigma) with a pH of 7.4
Hyaluronic acid (from human umbilical cord, Sigma) 3 mg/mL
Plasma proteins (from diluting fresh plasma) 1.5 mg/mL

Hyaluronic acid and plasma proteins in this solution interact synergistically with the combination binding more metal ion than either alone.

G. Serum

Serum is an important test fluid in that dissolution halftimes for some metal salts in serum are similar to excretion halftimes.[19] Human serum has been is purchased as a frozen liquid and has been filtered through a 0.1 um pore filter. Plasma is identical to serum except for the lack of fibrinogen and the presence or absence of fibrinogen would not be expected to appreciably affect solubility results.

D. pH of the Extraction Fluids

The extraction fluids were prepared as discussed. The pH for each fluid was as follows:

Fluid	Required pH
Gastric	1.5
Intracellular	4.5 – 5.0
Intestinal	7.4
Interstitial	7.4
Alveolar	7.4
Synovial	7.4

These pH values needed to be maintained throughout the entire extraction periods. The two acidic fluids (gastric and intracellular) were found to hold their required pH levels during storage and throughout the extractions. The remaining four fluids exhibited difficulties in this area. When these four solutions were prepared, the initial pH of the fluids was approximately 7.8± 2.0. Then, 2 N HCl was used to adjust the pH to 7.4. It was noticed that when the adjusted fluid was stored, the pH would continue to rise. Various waters (distilled, distilled/deionized, deionized, spring, and magnetic/carbon filtered) were used to prepare the solutions. It was found that lab deionized water could produce extraction fluids that would not drift once adjusted to a pH of 7.4. After it was determined that the pH levels for the four fluids could be maintained, the test compounds were introduced into the experiment. All compounds, except cobalt chloride and cobalt sulfate, caused the pH of the extraction solutions to drift up to 7.8 ± 2.0. For cobalt chloride and cobalt sulfate, the pH of the extraction solutions drifted downward. Some literature references claim that MOPS [3-(N-morpholino) propanesulfonic acid] buffers can be used to maintain pH levels in certain physiological solutions. A MOPS buffer was purchased and added to the interstitial fluid. Several extractions were performed. However, the buffer was unsuccessful at maintaining the required pH of 7.4. Next, a manual adjustment with HCl and NaOH was attempted at various intervals throughout the extraction. This proved to be tedious (especially for the longer extraction times). In an effort to try to keep the pH at 7.4 throughout the extraction, 5% CO_2 in nitrogen was

used to keep the pH of the solutions from rising. It was bubbled at a flow rate of approximately 50 cc/min. through the extraction solutions and was successful for maintaining the required pH levels. In cases where the compounds caused the pH to drop, manual pH adjustment was still performed with NaOH. Experiments were set up to compare manual pH adjustment to the CO_2 bubbling technique. The results showed comparable yet slightly higher results for those that were bubbled. This was expected since in the case of manual adjustment the average pH throughout the entire extraction would be greater than 7.4 and therefore not as much cobalt would be extracted. To be sure that all extractions were performed at the proper pH levels, the pH was checked prior to the extraction as well as after the extraction was completed. All pH levels and adjustments were kept on record.

E. Analysis

Flame Atomic Absorption Spectroscopy was the technique used to analyze all extraction fluids except serum. The instrumental parameters for the flame units are summarized in Table I.

Table I: Instrumental Parameters for Cobalt Determination using FL-AAS

Wavelength	240.7 nm
Slit Width	0.2 nm
Lamp Source	Hollow Cathode Lamp
Signal Type	AA-BG
Flame Type	Air/C_2H_2
Calibration	Non-linear

Working standards were prepared daily by making serial dilutions in each fluid from a 1000 ppm Fisher Scientific Cobalt Standard. The calibration consisted of a blank and three standards (0.5, 1.0, and 2.0 ppm). The calibration curve was established and optimized to obtain the greatest analytical range and best sensitivity possible. The correlation coefficient of the curve had to be >0.995 for the analysis to continue. The blank and standards were reanalyzed after calibration and on a continuing basis to verify the curve and to insure proper instrument performance. A Quality Control standard (0.05 ppm) was prepared in each fluid from a 100 ppm Spex Claritas Calibration Standard and was analyzed after calibration verification. The extract samples were brought to room temperature and mixed thoroughly prior to analysis. A number of dilutions were necessary for most of the extractions. Because of the high concentration of soluble cobalt, serial dilutions ranged from 1:100 to 1:10,000 to bring the values into the calibration range. For serum, the Zeeman Graphite Furnace Atomic Spectrometry technique was used for analysis. The instrumental parameters for the graphite furnace are summarized in Table II.

Table II: Instrumental Parameters for Cobalt Determination using:
Zeeman Graphite Furnace AAS

Wavelength	240.7 nm
Slit Width	0.2 nm
Lamp Source	Hollow Cathode Lamp
Sample Volume	20 μL
Chemical Modifier	5.0 μg Mg $(NO_3)_2$
Gas	Argon

Graphite Furnace

Step	Temp (°C)	Ramp (sec)	Hold (sec)	Gas Flow (ml/min.)	Read
Dry	100	5	30	250	
Dry	140	15	15	250	
Char	1400	10	20	250	
Atomize	2500	0	5	0	*
Clean	2600	1	3	250	

Working standards were prepared daily by making serial dilutions in serum from a 1000 ppm Fisher Scientific Cobalt Standard. The calibration curve consisted of a blank and four standards (10, 20, 50, and 100 ppb). The correlation coefficient of the calibration curve had to be >0.995 for the analysis to continue. The blank and standards were reanalyzed after calibration and on a continuing basis to verify the curve and to insure proper instrument performance. A Quality Control standard (5.0 ppb) was prepared in serum from a 100 ppm Spex Claritas Calibration Standard and was analyzed after calibration verification. The serum extract samples were brought to room temperature and mixed thoroughly prior to analysis. Dilutions of the extracts were made with 0.1% Triton X 100. As in the case of the flame analysis, dilutions ranging from 1:100 to 1:100,000 were necessary to bring the values into the calibration range. A solution was prepared that consisted of serum/0.1% Triton X 100 (1:10 by volume). All blanks, standards, quality control samples, and extracts were mixed 1:1 by volume with this solution prior to analysis so that the matrix would be consistent in all cases.

Results

Detection Limits and recovery

The method detection limits (MDLs) and percent recoveries for cobalt in all of the extraction fluids were established prior to any analysis of the sample extracts. The method detection limit was performed by preparing seven aliquots of each fluid at a cobalt concentration of 0.05 ppm. The aliquots were then analyzed by flame atomic absorption spectroscopy. A standard deviation (SD) was calculated and multiplied by the students' T value for seven replicates (3.143) to obtain the MDL. For serum, the MDL was performed by preparing seven aliquots at a cobalt concentration of 5.0 ppb. In this case, graphite furnace atomic absorption spectroscopy was used for the analysis due to the small volume of serum that was used for the extractions and to avoid possible analytical complications. The percent recoveries were determined by taking an aliquot of each of the extraction fluids and spiking each aliquot with cobalt to a concentration of 10 ppm. Each spiked fluid was taken through the entire 2 hour extraction procedure. The extracts were diluted into the calibration range and analyzed accordingly. The MDLs and percent recoveries are summarized below:

Extraction Fluid	MDL	% Recovery
Interstitial Fluid	0.044 ppm	102
Intracellular Fluid	0.001 ppm	101
Gastric Juice	0.012 ppm	95
Intestinal Juice	0.017 ppm	89
Alveolar Fluid	0.017 ppm	99
Synovial Fluid	0.017 ppm	86
Serum	2.47 ppb	111

Bioaccessibility of cobalt compounds

The maximum extractable cobalt levels, expressed as a percentage of available cobalt in the compound being tested, are presented in Table III. Results have been "standardized" to allow comparison from one compound to the next. We assume that if an assay was stopped because a material was >50% soluble, that the results are equivalent to 100% solubility. All of the cobalt carboxylates and salts tested were completely soluble in gastric juice equivalent: cobalt in these materials would be expected to be completely available for absorption if ingested. Elemental cobalt powder and cobalt oxide were also highly soluble in the low pH of the gastric juice and intracellular lysosomal equivalents and would be expected to provide absorbable cobalt if ingested or taken into a cell. The effect of pH was greater on the cobalt-tungsten carbide (hard metal) samples than on the cobalt spinel. Hard metal particles were more soluble in surrogate gastric juice and intracellular lysosomal fluid than the mixed-metal cobalt spinel. Both mixed-metal cobalt species demonstrated lowered bioaccessibility in the pH-neutral fluids compared to cobalt sulfate. The hard metal powders were, however, more soluble in human serum than was the cobalt spinel.

In the interstitial and alveolar equivalent fluids as well serum, the solubility of cobalt octoate was quite similar to that of cobalt sulfate heptahydrate. Cobalt bioaccessibilty for cobalt neodecanoate and cobalt naphthenate was less than for cobalt sulfate heptahydrate both in magnitude as well in time: the maximum solubility did not occur until after 72 hours of extraction. To better compare these carboxylates (e.g.cobalt driers) with cobalt sulfate heptahydrate, data for the first 24 hours (Table IV) were also analyzed. Testing against an intracellular lysosomal fluid equivalent was, however, similar to that of cobalt sulfate heptahydrate. If free particles of these cobalt carboxylates were taken up into cells they would be expected to be completely solubilized.

The salt cobalt carbonate was more stable in the pH neutral surrogate fluids than either cobalt chloride or cobalt sulfate heptahydrate. Most water-insoluble cobalt samples were more soluble in an intracellular lysosomal equivalent fluid with cobalt powder, cobalt oxide and carbonate being almost completely solubilized indicating they could provide absorbable cobalt if their particles were taken up by cells.

The cobalt spinel was sparsely soluble in all fluid equivalents and in serum. The Stellite alloy rod and powder were tested only in synovial fluid equivalent. Both physical forms were found to be almost completely stable over the entire extraction period**.**

Table III: Bioaccessibility of Cobalt Compounds
A. Driers (% of Available Cobalt Extracted)

Test Type	**Maximum Solubility (%)**		
	Cobalt Naphthenate	Cobalt Octoate	Cobalt Neodeconoate
Ingestion			
Gastric (pH 1.5)	>85.7	100	100
Intestinal (pH 7.4)	45.4*	50.8*	30.8*
Inhalation			
Alveolar (pH 7.4)	35.4*	>59.6	26.1*
Interstitial (pH 7.4)	40*	>80.4	43.1*
serum	42.9*	>66.9	46.6*
Intracellular (pH 4.5)	>79.1	100	>78.1

*maximum extraction level at 72 hours

B. Salts (% of Available Cobalt Extracted)

Test Type	**Maximum Solubility (%)**		
	$CoSO_4 \cdot 7H_2O$	$CoCl_2$	$CoCO_3$
Ingestion			
Gastric (pH 1.5)	100	>91.6	>92
Intestinal (pH 7.4)	>83.3	>79.4	4.1
Inhalation			
Alveolar (pH 7.4)	>51.4	>68	2.9
Interstitial (pH 7.4)	82.8	78.4	2.2
Serum	>81.7	>85	10.1
Intracellular (pH 4.5)	>83.3	>89.6	>96

C. Pigments (% of Available Cobalt Extracted)

Test Type	**Solubility (%)**	
	CoO	Cobalt Aluminum Spinel
Ingestion		
Gastric (pH 1.5)	>91.8	0.089
Intestinal (pH 7.4)	2.1	0.011
Inhalation		
Alveolar (pH 7.4)	2.4	0.015
Interstitial (pH 7.4)	9.9	0.006
Serum	19.9	0.095
Intracellular (pH 4.5)	92.4	0.089

D. Cobalt and Hard Metal Powder (% of Available Cobalt Extracted)

Test Type	Solubility (%)		
	Cobalt Tungsten Carbide pre-sintered	Cobalt Tungsten Carbide post-sintered	Cobalt Extra Fine HDFP
Ingestion			
Gastric (pH 1.5)	24.7	23.9	>67.3
Intestinal (pH 7.4)	15.1	6.1	3.7
Inhalation			
Alveolar (pH 7.4)	2.6	3.6	4.8
Interstitial (pH 7.4)	8.3	12.4	4
Serum	21.7	14.7	11.3
Intracellular (pH 4.5)	25.9	26.7	>91.1

E. Cobalt Chrome Alloys (% of Available Cobalt Extracted)

Test Type	Solubility (%)	
	Stellite 21 Powder	Stellite Rods
Synovial (pH 7.4)	0.0018	0.0013

Table IV: Bioaccessibility of Cobalt Driers (% of Available Cobalt Extracted) at 2 or 24 hours

Test Type	Maximum Solubility (%)			
	Cobalt Naphthenate	Cobalt Octoate	Cobalt Neodeconoate	Cobalt Sulfate Heptahydrate
Inhalation				
Alveolar (pH 7.4)	21.3	>59.6	19.8	>51.4
Interstitial (pH 7.4)	40	>80.4	16.8	82.8
Serum	20.6	>66.9	28.6	>81.7
Intracellular (pH 4.5)	>79.1	100	>78.1	>83.3

Discussion

In evaluating the data, the overall precision was very good. There were some factors that influenced the indeterminate error that is inherent in the methodology. First, because of the high concentration of cobalt in the final extracts of some assays, very large dilutions had to be made prior to atomic absorption analysis. When dilutions of this magnitude are performed, any small error is multiplied as the dilution factor is taken into account in calculating the final result. This increase in standard error will increase the minimum detection limit by a factor of about 10. Second, in the cases where the pH had to be maintained at 7.4, it was found that even a slight pH variation could have a profound effect on the cobalt result. This was controlled by closely monitoring pH during the incubation and adjusting pH as needed. Third, some compounds did not reproduce very well in certain fluids. In these cases the number of replicates was increased.

The aims of this study were two-fold. The first was to compare the bioaccessibility of selected species of cobalt (e.g. salts, oxides, organic, or mixed-metal compounds) in human surrogate tissue fluids and human serum. The second aim was to initiate studies to experimentally validate an *in vitro* methodology (e.g. cobalt bioaccessibility testing) that might provide an estimate of bioavailability in the assessment of dose from exposure to various species of cobalt compounds. Generally the dose is unknown in human exposures to metal compounds making the assessment of safe exposure levels difficult.

One of the major concerns for exposure to cobalt compounds is from inhalation. [20, 21, 22] In occupational and consumer-use settings humans may be exposed to a variety of cobalt species from elemental cobalt powders to inorganic water-soluble and water-insoluble salts , organic salts, or mixed-metal compounds such as cobalt tungsten carbide or mixed-metal cobalt spinels. The use or application of paints, coatings, and some art and craft materials also provide opportunities for both occupational and consumer cobalt exposures**.** Cobalt oxide is used in ceramic glazes and cobalt aluminum spinel is a colorant used in paints. Inhalation exposures can occur during mixing of dry glazes or paints, sanding or during spray application. Cobalt driers (e.g. cobalt carboxylates**)** are used in various oil and alkyd paints and mediums. Although these driers are not manufactured in respirable particle form, inhalation exposures can occur during spray applications and sanding of painted surfaces.[20, 23, 24] Exposures to cobalt driers only occur when they are in a paint or resin matrix. Once dried the bioaccessibility of cobalt in a paint film or resin may be decreased further by a factor of 10 or more, depending upon the matrix.[40]

Four measures were used to evaluate the bioaccessibility of cobalt species (e.g. cobalt compounds) if inhaled, using synthetic equivalents of alveolar fluid, interstitial fluid, intracellular lysosomal fluid, and human serum. In these fluids the selected cobalt species showed variations in bioaccessibility. Two cobalt carboxylates (neodeconoate and napthenate), elemental cobalt powder and cobalt oxide appear to have different solubility (lower) than cobalt sulfate, chloride, or cobalt octoate in the pH neutral (e.g. pH 7.4) lung fluid equivalents. Also, the cobalt carbonate salt was much less soluble in

the lung surrogate fluids than were the sulfate and chloride salts. The mixed-metal species, cobalt aluminum spinel and the cobalt tungsten carbides were also less soluble in the pH 7.4 lung fluid equivalents. The impact of pH on the bioaccessibility of most all of the cobalt species (excluding the spinel) was the most dramatic as indicated by their enhanced solubility in intracellular lyosomal fluid. Based on the results, particles of these cobalt compounds if taken up by cells would be expected to be bioavailable within the cell. The increased acidity had a lesser but still variable effect on the mixed-metal cobalt species. Hard metal (cobalt-tungsten carbide) particles might be expected to provide more bioavailable cobalt if taken up by cells than cobalt aluminum spinel. These data support the idea that the metal compound (e.g. metal speciation) is an important determinate of metal bioavailability in human tissue fluids.

Two factors affect whether or not cobalt will be absorbed into lung tissues: solubility and particle size.[25, 26, 27] In healthy, non-smoking individuals, up to 90% of particles depositing in airways are cleared from the respiratory tract in 24 hours. The removal of particles from the terminal bronchioles and alveoli may take considerably longer.[28, 29, 30, 31, 32] In assessing, for example, a bioavailable dose from exposure to particles of cobalt neodecanoate or napthenate one scenario might consider that since 75-80% of these particles were insoluble during the first 24 hours, the major fraction of these inhaled cobalt compounds might be effectively cleared and therefore unavailable for uptake into tissues of the respiratory tract. The cobalt aluminum spinel was greater than 99% insoluble during this time suggesting cobalt from this pigment would not be acutely bioavailable for absorption into lung tissues**.** Risk of adverse effects will also be dependent upon the pathological state of the lungs, as well as, amount of cobalt compound inhaled, which, in turn, will be dependent on application and ventilation parameters.

Oral over-exposure to water-soluble cobalt compounds in humans has been associated with an increased risk for the development of goiter, polycythemia, and under specific conditions, cardiomyopathy.[33, 34] The level of health risk varies with the amount of soluble cobalt ingested and predisposing factors, such as diet and alcohol consumption. The selected cobalt carboxylates, cobalt salts, cobalt oxide, and elemental cobalt metal powders are readily soluble in a gastric juice surrogate. The hard metal powders were much less soluble. Ingestion of dusts or liquids containing any of these cobalt compounds would, however, be associated with exposure to some bioavailable cobalt. Cobalt aluminum spinel, on the other hand, showed little solubility in a gastric juice surrogate or synthetic small intestinal juice. Ingestion of paints or glazes containing this or similar sintered cobalt pigments would be expected to present less risk of thyroid or heart problems than the more water-soluble cobalt compounds. Employing gastric and intestinal fluid surrogates for the assessment of bioaccessibility of cobalt species should be useful in predicting the bioavailability of cobalt compounds and determining doses related to health risks caused by their ingestion.

Case studies have associated the use of metal joint prostheses with the occurrence of local sarcomas. IARC has reviewed these studies and were unable to classify implants containing cobalt as a cancer risk to humans.[35] A more recent cohort study failed to find any relationship between hip prostheses containing cobalt and an increased risk of bone

or connective tissue tumors.[36] If a metal were to add to the risk of prostheses-related tumors, one would expect that synovial fluid would solubilize the metal. Our tests of the particulate and massive stellite alloys show the low solubility of these alloys in a fluid chosen to be equivalent to joint fluid. Other studies have shown that implants do provide bioavailable cobalt over a period of use-time. The increases are managed, however, by transient increases in the elimination of bioavailable cobalt from tissues via the urine.[37] We believe it is unlikely that cobalt solubilized from these alloys would add to risk of either local or distant tumors.

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