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Importance of preanalytical factors in measuring Cr and Co levels in human whole blood: contamination control, proper sample collection, and long-term storage stability.

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Abstract

A number of errors with potentially significant consequences may be introduced at various points in the analytical process which result in skewed, erroneous analytical results. Precautionary procedures such as contamination control, following established sample collection protocols, and having a complete understanding of the long-term stability of the elements of interest can minimize or eliminate these errors. Contamination control is critical in quantification of Cr and Co in human whole blood. Cr and Co levels in most biological samples are low, but these elements occur naturally in the environment and are often found in commercial and consumer products, which increases the risk of contamination. In this paper, we demonstrated that lot screening process in which we pre-screen a sub-set of manufactured lots used in collecting, analyzing, and storing blood samples is a critical step in controlling Cr and Co contamination. Stainless steel needles are often utilized in blood collection but are considered a potential source of introducing metal contamination to the patient sample. We conducted two studies to determine if there is a possibility of Cr or Co leaching into the human whole blood from the needles during blood collection. We analyzed blood collected from 100 donors and blood collected in-vitro in the laboratory from designated vessel containing spiked blood with higher levels of Cr and Co. Two blood tubes were consecutively collected through one needle. In both studies, Cr and Co concentration levels in the two consecutively collected tubes were compared. Based on the results from donor and in-vitro blood collection studies, we concluded there was no Cr and Co leaching from the limited sets of stainless steel needles used in these studies. Further, we demonstrated that Cr and Co human whole blood samples are stable for one year stored at temperatures of −70°C, −20°C, and 4°C, and six months at room temperature.

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DISCLOSURE STATEMENT

The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services or the U.S. Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the U.S. Centers for Disease Control and Prevention.

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Keywords

chromium; cobalt; metal-on-metal hip implants; biomonitoring; long-term storage; contamination control; blood; needles; screening; collection devices; preanalytical factors; sample collection; sample handling

INTRODUCTION

Chromium (Cr) and cobalt (Co) are minerals required as dietary supplements in trace amounts; however, when present in high concentrations both metals have toxic effects. Exposure to high levels of Cr and Co has been linked to both cutaneous and extracutaneous allergic reactions, cellular toxicity, cancer, and inflammation [1–5]. In the past, overexposure to both metals has been associated with environmental and industrial exposure [6]. Recently, doctors have become concerned about health effects caused by the toxic levels of Cr and Co in the blood stream released by metal-on-metal (MoM) bearing surface and metallic junctions of hip implants [7, 8]. Monitoring blood Cr and Co concentrations in patients that may have degraded MoM hip replacements has become more urgent so that the medical community has a simple test to determine the need for possible follow-up medical interventions. To this end, we developed an analytical method capable of rapidly and accurately quantifying Cr and Co in human whole blood by inductively coupled plasma - kinetic energy discrimination - mass spectrometry (ICP-KED-MS) [9]. Currently, our laboratory uses this method in the National Health and Nutrition Examination Survey (NHANES)[10] to gather population data about Cr and Co exposure in the U.S. population, in participants that are 40+ years old. The NHANES data values will help establish Cr and Co reference values for baseline levels in blood. Knowing U.S. baseline population exposure levels for Cr and Co will help researchers and clinicians to more accurately correlate adverse health effects with specific exposure levels.

The validity of analytical results depends not only on the instrumentation and method, but also on the preanalytical steps, such as contamination control, following established correct sample collection protocols, and understanding analyte storage stability [11, 12]. A critical step in ensuring the quality of our laboratory's measurements is minimizing the levels of contamination introduced to prevent adverse effects on analytical process. Researchers have long recognized the importance of strictly controlling potential sources of contamination to the extent possible in clinical elemental analysis [11, 13–16]. However, information on contamination from the devices that are commonly used at these stages is still lacking. Since our laboratory has collected data that refutes claims of certain devices being completely free of trace element contaminants, we screen sub-sets of manufactured lots of all items that come into contact with biologic samples during sample collection, storage, and analysis. We refer to this screening process as lot screening[17]. By integrating lot screening into the first phase of our analytical process, we reduce the likelihood of obtaining falsely elevated analytical results attributed to contamination in such items as needles and evacuated tubes used for blood collection or cryovials used for blood storage. The alternative to lot screening would be acid washing each device or container before use [18]. Since our laboratory typically conducts large national surveys and biomonitoring studies, acid washing all individual items

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involved in sample collection, storage, and analysis is not feasible. Furthermore, with some items used in blood metals analysis such as evacuated blood tubes filled with EDTA, acid washing is not feasible because the process would remove the anti-coagulant.

Airborne contamination is as important as contamination from containers [11, 15]. In principle, the sample preparation procedure should be carried out inside of a laminar flow hood Class 100 or better [18]. In fact, our laboratory uses a dedicated class 10,000 clean room to perform elemental analysis[17, 19], after sample preparation is continued in a US Class 10 (ISO class 4)biosafety cabinet (BSC).

Blood sample collection is another important step in the preanalytical process. To ensure test reliability, well-trained staff should conduct blood collection using appropriate devices[16]. Trace element contamination from blood collection devices such as syringes, collection tubes, and needles is possible [11, 13, 20, 21] and may potentially introduce additional levels of the elements of interest to the sample, thereby creating a bias. Current guidelines recommend using a plastic intravenous (IV) cannula rather than a stainless steel needle when collecting blood samples for measuring trace elements [16, 22]. However, plastic IV cannulas are more difficult to handle, and many patients do not have veins suitable for using plastic cannulas [21, 23]. Giving clear recommendations on blood sampling techniques [21] for trace metal analysis is important; however, guidance can be contradictory. Some studies in scientific literature have shown evidence of Cr and Co contamination from needles [11–13, 15, 24–26], but others have shown no conclusive evidence of significant contamination [21, 27–29]. Hodnett et al. found no significant difference in concentrations of Cr obtained by different methods of blood sampling (standard needle, “butterfly” winged needle, or plastic cannula) [29] while Penny and Overgaard showed some evidence of Cr contamination from stainless steel needles compared to plastic IV cannulas [21]. There are fewer cases of Co contamination since stainless steel needles contain smaller quantities of Co [29]. Barry et al. demonstrated that Co and Cr levels were not significantly greater in purge samples (direct contact with needle) than reserve samples (no needle contact) [28]. Although when the contamination is not clinically significant, it still may be potentially significant from a population biomonitoring point of view. In our laboratory, for the purposes of NHANES sample analysis, we measure Co and Cr from the second venipuncture tube collected in order to reduce the possibility of sample contamination from the stainless steel needles.

Due to lack of clear evidence in literature on contaminations caused by stainless steel needles during blood collections, we conducted a limited study to evaluate if Cr and Co leaches from a single lot and model stainless steel needles during blood collection. Our study consisted of analyzing blood from two evacuated blood tubes that were collected by personnel from Tennessee Blood Services Corporation (TBSC) consecutively through one needle from 100 human donors. This blood was anonymously collected by TBSC with no personal identification provided to us. TBSC collected 200 tubes of blood using a total of 100 needles. For each donor, we compared Cr and Co concentrations between the 1st and 2nd tube of blood collected. Further, in our laboratory we conducted an in-vitro study to simulate a real-world blood collection scenario. We used a pooled blood source that was spiked with Cr and Co at levels of 1.5 μg/L and 0.9 μg/L, respectively which were similar to the 95th percentile from the 2015-2016 NHANES cycle (Cr - 1.08 μg/L and Co - 0.4 μg/

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L)[30]. We filled 200 evacuated blood tubes using 100 needles. In both studies, we compared Cr and Co levels in two consecutively collected tubes.

Additionally, this paper aims to fill some existing gaps in scientific literature on the long-term storage stability of Cr and Co human whole blood samples. Blood analysis immediately after sample collection is rarely possible. Therefore, understanding the stability of these Cr and Co whole blood samples between blood collection and analytical quantification is important. The amount of time to transport samples from the field to a laboratory for analysis can vary significantly, from hours to days or even weeks, and storage temperatures during transportation are sometimes not ideal [11]. Also, for population surveys and community exposure evaluations, large numbers of samples may need to be analyzed. Those samples undergo many logistic steps and long storage periods before laboratory analysis [11]. We need to confirm that changes in temperature over a period of 1 year do not cause whole blood to clot or become viscous due to evaporation, which would make it impossible to correctly quantify Cr and Co analytes. Additionally, there is a possibility of Cr and Co adhering to storage container or precipitating into a protein globulin which could make it insoluble and introduce an error to the analysis. For these reasons, ensuring that the integrity of samples is not compromised over time at different temperatures is vital.

Only a few published papers mention the stability of Cr and Co concentration levels in blood samples [14, 28, 31]. Barry et al. showed Cr and Co stability in blood at −20°C between 0 to 29 months [28]. Lindberg-Larsen and Penny found no concentration reduction in the mean levels of Cr and Co comparing testing results from blood frozen immediately after collection with blood frozen after 4 and 30 days [31]. To get a better understanding of behavior of Cr and Co whole blood samples, we conducted a study monitoring the concentrations of these analytes in human blood at four different temperatures (−70°C, −20°C, 4°C, and room temperature [RT]) over one year.

The first objective of this paper is to introduce the lot screening procedure as it pertains to blood Cr and Co sample collection and its importance in controlling contamination in trace elemental analysis. In this respect, we provide examples of devices screened in our laboratory and their susceptibility to contamination. To date, there has not been any published data on clinical devices susceptibility to Cr and Co contamination and on how critical pre-screening procedures are in minimizing or preventing this contamination. The second objective is to provide results of the study where we tested two consecutively drawn blood tubes through one needle from each of 100 donors (blood samples provided by TBSC) in order to determine whether any Cr and Co leaches from the particular lot of stainless steel needles used in this study during the blood collection procedure. Further, we replicated this blood collection study in-vitro in our laboratory using Cr and Co spiked blood to evaluate contamination of samples containing higher concentration levels of Cr and Co. To the best of our knowledge, this is the only study analyzing such a large amount donor and in-vitro blood samples to evaluate Cr and Co leaching during sample collection from stainless steel needles using this innovative experimental approach. The third objective is to present a long-term stability study of Cr and Co human whole blood samples over the course of one year, stored at four different temperatures: −70°C, −20°C, 4°C, and RT. It is necessary to point that this is the first extensive study monitoring stability for Cr and Co in human blood. For these

analyses, we used ICP-KED-MS to quantify Cr and Co in human whole blood and aqueous solutions. We conducted statistical analysis of the data to develop a comprehensive understanding of changes and trends in Cr and Co concentrations.

EXPERIMENTAL

A detailed description of the Cr and Co analytical method and sample preparation procedures can be found in the method paper recently published by our group[9]. Therefore, in this article we only provide a brief summary of the experimental protocols followed.

Reagents

We prepared all reagents using laboratory deionized water (18 MΩ·cm, measurement of the resistance of DI water) filtered in-house using an Aqua Solutions Water Purification system (Jasper, GA). Sample diluent, carrier, and rinse solutions consisted of 0.01% ammonium pyrrolidine dithiocarbamate (APDC, Fisher Scientific, Waltham, MA), 0.4% (v/v) tetramethylammonium hydroxide (TMAH, 25% in H_2O, Alfa Aesar, Ward Hill, MA), 1% ethanol (Pharmco Products Inc., Brookfield, CT), and 0.05% Triton X-100 (Sigma Aldrich, St. Louis, MO). We added 20 μg/L of gallium (Ga) and scandium (Sc) internal standards (Inorganic Ventures, Christiansburg, VA) to diluent and carrier solutions. We used single element stock standards of Cr and Co from various sources (Inorganic Ventures, Christiansburg, VA; National Institute for Standards and Technology (NIST), Gaithersburg, MD; SPEX CertiPrep, Metuchen, NJ; High-Purity Standards, Charleston, SC to prepare calibration standards in 2% v/v nitric acid (HNO_3, environmental grade, GFS Chemicals, Columbus, OH) and 1% v/v double distilled hydrochloric acid (HCl, 30–35%, GFS Chemicals, Columbus, OH). All calibration standards are traceable to the NIST. We purchased human whole blood with ethylenediaminetetraacetic acid (EDTA) from Tennessee Blood Services Corporation (TBSC), Memphis, TN for matrix matching of the calibration curve and preparing in-house quality control (QC) material. For matrix matching, we used non-spiked blood, referred to as "base blood" in this paper. The sample blood tubes collected from 100 donors for Cr and Co leaching from stainless needles study were purchased from TBSC.

Sample preparation

For blood sample analysis we used a calibration curve covering analyte concentrations up to 15 μg/L consisting of five calibrators with concentrations of 0.5, 1.5, 5.0, 7.5, and 15 μg/L [9]. To prepare a set of calibrators, we used 0.25 mL of the standard, 0.25 mL of base blood, and 4.5 mL of diluent. We prepared in-house QC samples, long-term stability samples, and the 1st and 2nd blood tube samples by mixing 0.25 mL of the blood sample, 0.25 mL of water, and 4.5 mL of diluent. For the lot screening procedures, we used different calibration levels (0.5, 1, 2.5, and 5.0 μg/L) since the required concentration range is smaller. To prepare a set of these calibrators, we used 0.25 mL of the standard and 4.5 mL of diluent. For the lot screening sample, we used 0.25 mL of aqueous sample (screening solution) plus 4.5 mL of diluent.

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In-house QC material

We prepared two sets of pooled samples using human whole blood (EDTA disodium salt used as anti-coagulant) purchased from the TBSC (Institutional Review Board [IRB] TB-12-01, expiration November 18, 2020). We distinguished these as the low blood (A) pool, spiked with low concentrations of Cr and Co analytes, and the high blood (B) pool, spiked with higher concentrations of Cr and Co. Table SI in Supplemental Information (SI) summarizes the calculated concentration results for the two pools over 50 analytical runs on two different instruments and by two analysts. Calculated Cr and Co concentrations in pool A are 2.58 μg/L and 1.66 μg/L and for pool B are 12.26 μg/L and 8.89 μg/L, respectively.

We reserved specific portions of each of the A and B pools to use as long-term stability study samples. We used additional samples from each pool with each analytical run as bracketing quality control (QC) material samples. For our quality assurance and quality control programs, patient samples (or any samples) are bracketed with this QC material. If bracketing QC concentration falls outside of its 3SD range, the analytical run is marked as "fail," and the sample analysis is repeated. In practice, we use more sophisticated evaluation criteria based on modified Westgard rules[32, 33]. In this paper, we refer to 3SD range of QC material as a "quality assurance limit" (QAL).

Instrumentation

We used a Thermo® iCAP™ Qa (upgraded to an iCAP Qc) ICP-MS with collision cell (QCell™, Thermo Fisher Scientific, Waltham, MA) equipped with a peristaltic pump, a PFA-ST MicroFlow Nebulizer, a peltier-cooled cyclonic spray chamber, a 2.0 mm quartz injector, platinum sampler cone, platinum skimmer cone, and skimmer cone insert 3.5 mm for all experiments. The instrument parameters can be found in our preceding Cr and Co method paper [9]. We used an SC-4DX FAST autosampler (Elemental Scientific Inc., Omaha, NE) for the uptake of diluted blood samples for analysis and control of the FAST sample introduction timing. See our method paper for detailed information on the FAST method [9]. Our laboratory used > 99.999% argon (Specialty Gases Southeast, Airgas, Atlanta, GA) for the plasma and nebulizer gases. We operated the collision cell in KED mode at 5 mL/min helium gas flow (99.999% grade, Airgas South, Kennesaw, GA). We prepared final dilutions (calibrators, QC, and unknown samples) for analysis using a Hamilton Microlab 625 advanced dual syringe diluter (Hamilton Company, Reno, NV), equipped with a 10 mL dispensing syringe and a 500 μL sampling syringe.

METHODS

Lot screening procedure

The Cr and Co lot screening procedure is to test 50 devices from a manufacturing lot to determine if the lot is suitable for use in collecting, analysis, and storage of blood samples. To prepare a device for Cr and Co lot screening, we either put screening solution in the device (e.g. vacutainers and cryovials), or we pass a screening solution through the device (e.g. needles, syringes). For Cr and Co lot screening we use water as a screening solution. Before use we test the water to make sure there are no detectable levels of Cr and Co. The volume of screening solution is determined by the amount of blood sample that would either

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pass through the device or be stored in a container. The contact time of the screening solution with each device depends on the type of device. For instance, for cryovials, we uncap the vials, add screening solution, and recap the vial. We invert the 25 odd-numbered cryovials and let the devices sit overnight (8 to 12 hours)[17]. If contamination is present, this allows us to determine whether it comes primarily from the vial (the even units that were left upright), the cap (all inverted vials), or both. A variety of types of devices are screened using this methodology. With needles, we pass the screening solution through the needle into a prescreened evacuated blood tube, thus the contact time with the screening solution is identical to the contact time of blood being passed through a needle[17].

For Cr and Co lot screening we use the same instrumentation and quantification method as for analysis of blood samples. We prepared QC for the lot screening process by diluting NIST 1640a standard reference material (NIST, Gaithersburg, MD) with water using a 1 + 9 dilution (1 mL of NIST 1640a plus 9 mL of DI water), referred to hereafter as "1640a_10x." QC passing criteria can be found in Table SII. If Cr or Co 1640a_10x concentrations are not within ± 10%, the analytical run fails and must be repeated.

For the Cr and Co lot screening procedure; we allow no more than 5% of the units to be defective in an acceptable lot with a 90% confidence interval[17]. We use Equation 1 to determine criteria for the maximum (max) allowable contribution to total concentration of the analyte that can be present in the device or container for it to pass lot screening. We consider a lot failing if it has more than one result for the analyte of interest above the max allowable contribution. If no devices are above the max allowable contribution, we consider the lot acceptable for use in the Cr and Co biomonitoring method. If only one device of the screened 50 fails, we use our scientific knowledge to decide whether this failure should be considered an outlier.

$$\begin{aligned} & Max\ allowable\ contribution \\ & = \frac{(expected\ population\ mean) \times (max\%contribution) \times (volume\ sample\ in\ device)}{(volume\ screening\ solution)} \end{aligned} \quad \text{Equation 1:}$$

The "expected population mean" represents the expected mean concentration of the analyte of interest in the population to be studied. We use 0.151 μg/L and 0.56 μg/L for Co and Cr, respectively. The Co value is based on the geometric mean for blood Co from the 2015-2016 NHANES cycle[30]. Since Cr does not have a geometric mean due to the majority of the values being below the LOD, we use 50th percentile based on 107 samples analyzed by our laboratory in 2014. The "max percent contribution" is the percentage of the analyte allowed to be present in the device tested, in our laboratory we set that value to 10%. The "volume sample in device" stands for the amount of sample that will either pass through the device or be stored in the container tested. The "volume screening solution" is the amount of screening solution that passes through the device or is aliquoted in the storage container during screening. If the calculated "max allowable contribution" is below the limit of detection (LOD) of the method (Cr - 0.41 μg/L and Co – 0.06 μg/L), then the "max allowable contribution" is equal to 1.5 times LOD of the method (Cr – 0.62 μg/L and Co – 0.09 μg/L).

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Blood collection study

Donors—We received 200 samples collected by TBSC personnel from 100 donors ages 40+ (Institutional Review Board [IRB] TB-12-01, expiration date November 18, 2020). Donor details can be found in Table SIII . We selected the age of 40 and older to be representative of the population that potentially have MoM hip implants. This age range is the same range we selected to establish Cr and Co baseline levels for the U.S. population in the NHANES. TBSC collected two samples (two filled evacuated blood tubes) per donor: the 1st and 2nd blood tubes were collected consecutively using one needle/luer adaptor. We received two 4 mL K_2EDTA venous blood collection tubes (Becton Dickson, Franklin Lakes, NJ) of human whole blood from 100 donors (200 samples). Samples were drawn from each donor's arm using a multi-sample blood collecting needle (Exel International, Medline, Mundelein, IL) with an multi-sample luer holder (Exel International, Medline, Mundelein, IL). The collected samples were stored at −70°C until analysis. All the 1st blood tubes were analyzed in random order over the period of 2 days and 2nd tubes were analyzed in random order over the following 2 days.

In-vitro—In addition to analyzing blood samples collected from donors, we performed a controlled in-vitro study in our laboratory to simulate a real-world blood collection scenario but used blood spiked with higher levels of Cr and Co. We spiked base blood purchased from TBSC with single element Cr and Co stock standards (Inorganic Ventures, Christiansburg, VA). The spiked base blood was stirred overnight and subsequently analyzed to obtain native Cr and Co concentrations. The blood had Cr and Co concentrations of 1.5 µg/L and 0.9 µg/L, respectively. These concentrations were chosen to be more representative of blood concentrations of the US population at the 95th percentile from 2015-2016 NHANES (Cr – 1.08 µg/L and Co- 0.4 µg/L)[30]. We used 4 mL K_2EDTA purple top tubes (Greiner Bio-one GmbH, Monroe, NC) to collect blood through safety blood collection sets plus luer adapter (21G ¾', tubing length 12" Greiner Bio-one GmbH, Monroe, NC) from a triple washed plastic container filled with spiked blood. Two tubes were filled consecutively through one needle (200 tubes were filled, and 100 needles used). All samples were analyzed in random order.

A paired sample t-test[34] was conducted to compare mean Cr and Co concentrations from 1st to 2nd blood tubes to determine any statistically significant difference between them in both donor and in-vitro studies. Collection devices used for these two studies were not lot screened by our laboratory in order to imitate typical collection without screening.

Long-term stability study

We used human whole blood based in-house QC material to assess the long-term storage stability of Cr and Co whole blood samples. We stored aliquots of the pooled materials (A and B) for one year at four different temperatures: −70°C, −20°C, 4°C, and RT. We evaluated −70°C and −20°C, since these are established long-term storage temperatures [35, 36]. We evaluated the recommended short-term storage temperatures of 4°C and room temperature (RT) to account for cases when samples are left at RT during sample processing or when refrigerators are not available. We chose not to evaluate samples stored at an elevated temperatures because based on a previous blood metals stability study for other

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elements, we know that the blood matrix becomes viscous or dry after two weeks when stored at 37°C [37]. Aliquots of the A and B pools were analyzed at 1 day; 1, 2, 4, and 6 weeks of storage; and at 2, 4, 6, 8, 10, and 12 months of storage (“E” stands for time event. E1=1 day; E2=1 week; E3=2 weeks; E4=4 weeks; E5=6 weeks; E6=2 months; E7=4 months; E8=6 months; E9=8 months; E10=10 months; E11=1year). At each time event, we analyzed three aliquots of A and B in duplicate per each temperature condition. After original analysis, we did not choose these aliquots for subsequent analysis. We bracketed each stability analytical run with QC material. We implemented the 3SD range quality assurance limit (QAL) (Table SI) to examine the long-term stability of A and B aliquots. If Cr and/or Co concentrations in A and B stability samples fall outside of our established QAL, Cr and/or Co whole blood samples are not considered stable. If they fall within our QAL, these samples are considered stable.

For statistical analysis, first we used a general linear regression model to fit the long-term stability data. The model included time (categorical) and temperature (categorical) as covariates and the measurement value was the outcome. Further, we evaluated the study results using the equivalence test [38]. We used Cr and Co concentration values from −70°C as the “reference” and compared Cr and Co concentration values from temperatures (−20°C, 4°C, RT) against the values at −70°C. The hypotheses were as follows:

$$H_0 \ \ \mu_{Test}/\mu_{Ref} < 0.8 \text{ or } \mu_{Test}/\mu_{Ref} > 1.25$$

$$H_1 \ \ 0.8 \le \mu_{Test}/\mu_{Ref} \le 1.25$$

with μ_{Test}: Cr and Co concentration values at −20°C, 4°C, and RT and μ_{Ref}: values at −70°C.

Next, we used Cr and Co concentration values from day 1 of storage (E1) as the “reference” and compared the Cr and Co concentration values from the other 10 events (E2=1 week; E3=2 weeks; E4=4 weeks; E5=6 weeks; E6=2 months; E7=4 months; E8=6 months; E9=8 months; E10=10 months; E11=1year.) against the values in E1. The hypotheses were as follows:

$$H_0 \ \ \mu_{Test}/\mu_{Ref} < 0.8 \text{ or } \mu_{Test}/\mu_{Ref} > 1.25$$

$$H_1 \ \ 0.8 \le \mu_{Test}/\mu_{Ref} \le 1.25$$

with μ_{Test}: values at 1, 2, 4, 6 weeks and 2, 4, 6, 8, 10, and 12 months and μ_{Ref}: value at 1 day.

If the ratio of μ_{Test}/μ_{Ref} was less than 1, we tested the hypothesis of $\mu_{Test}/\mu_{Ref} < 0.8$. If the ratio was greater than 1, we tested the hypothesis of $\mu_{Test}/\mu_{Ref} > 1.25$. If the p-value was less than 0.05 for hypothesis testing, we rejected the null hypothesis (H_0) and accepted the alternative hypothesis (H_1:0.8 μ_{Test}/μ_{Ref} 1.25), meaning that the test storage condition

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results were statistically equivalent (no difference) to the reference storage condition results. We conducted the statistical analysis using SAS software [34].

RESULTS AND DISCUSSION

Lot screening

Since developing the Cr and Co biomonitoring method in 2014 [9], we have screened 156 manufacturing lots to determine their suitability for laboratory and field use (data collected from November 2014 through January 2018), Table 1. Out of these lots, 22 failed for Cr and/or Co. Data in Table 1 reveal interesting trends and failure percentages for different types of devices. For all the screened devices our calculated “maximum allowable contribution” was below the LOD, thus we used 1.5 times LOD values of Cr - 0.09 μg/L and Co - 0.62 μg/L. We tested 58 manufactured lots of evacuated blood collection tubes and obtained a 21% failure rate (10 lots failed for Co (17%) and 4 for Cr (7%)). Evacuated tubes are widely used as blood collection devices. Since the possibility of contamination is high, prescreening them prior to use is particularly important. For both Cr and Co, we tested 6 lots of transfer pipettes with a 17% failure rate (1 lot) and 11 lots of polypropylene tubes with an 18% failure rate (2 lots). We analyzed only a few lots of these devices, but failures occurred, demonstrating the need for screening before use. We analyzed one lot of microvettes (fingerstick blood collection device) which failed for Cr. Six lots of alcohol pads were analyzed and two lots failed for Cr and Co. From our experience with other metal screening methods, alcohol pads often have unacceptable levels of metal contamination. Needles had 8% failure rate; out of 49 lots screened, four lots failed. Cryovials (16 lots), luer-adapters (7 lots), and syringes (2 lots) had no failures. The highest individual screening results from the screened lots from 2014 through 2018 for Cr were needles (4.62 μg/L), vacutainers (2.34 μg/L), and microvettes (1.54 μg/L) and for Co were vacutainers (0.92 and 0.89 μg/L) and alcohol pads (0.67 μg/L) , Table SIV. These preliminary results reveal that a significant number of lots had failures, thus supporting the necessity of screening all devices that come in contact with a patient sample, either in the laboratory or in the field, to reduce the likelihood of contaminating a patient sample

Blood collection study

In general, Cr and Co contamination of human blood samples collected using stainless steel needles is considered low; however, when measuring very low concentrations of these metals for establishing reference ranges, even small levels of contamination could have an effect [11, 15, 21, 24, 27–29]. Therefore, we conducted a study comparing Cr and Co concentration in 1st and 2nd consecutively collected blood tubes using the one needle device to detect if there is any Cr and Co leaching out of the needles during blood draw procedure.

Our laboratory analyzed 200 samples from 100 donors collected by TBSC. TBSC personnel collected two sample tubes per donor: 1st and 2nd blood tubes were collected consecutively using the same medical set up (needle/luer adaptor), Figure 1a and b show graphic illustration of the Cr and Co concentration difference between 1st and 2nd blood tubes in patient samples (actual concentrations can be found in Table SIII). Figure 1a shows slightly higher results for Cr concentrations for the 2nd blood tube over the 1st in some donors,

making the mean concentration difference negative (Table 2). Figure 1b shows the difference in Co concentrations between the 1st and 2nd blood tubes, there is no visual trend.

After the data collection, we performed paired sample t-test analysis to determine any statistically significant difference in Cr and Co concentrations between the 1st and 2nd blood tubes (Table 2). The paired sample t-test produced, p-values below 0.05, indicating there is a statistically significant difference between Cr and Co concentrations in 1st and 2nd blood tubes. The mean concentration difference between the 1st and the 2nd blood tube for Cr was negative 0.191 μg/L, meaning that the average Cr concentration in the 2nd blood tube was higher than in the 1st (Table 2). If Cr was leaching out of the stainless steel needle, we would expect mean Cr concentration in the 1st tube to be higher than the concentration in the 2nd, however, we saw the opposite effect. Out of 100 samples analyzed for the 1st blood tube, Cr concentration in 90 samples (90%) was below the method's LOD limit (0.41 μg/L). For the 2nd blood draw, out of 100 samples analyzed, Cr concentration in 78 samples (78%) was below the LOD limit. For Co, the average concentration difference was positive 0.024 μg/L, indicating the Co mean concentration for the 1st tube was higher than the Co concentration for the 2nd tube. However, since the concentration difference is very small 0.024 μg/L compared to the LOD for Co being 0.06 μg/L, we think this difference is possibly due to variations in instrumental response, sample preparation, or other experimental parameters and not leaching. Further, we consider the Cr and Co differences (0.191 μg/L and 0.024 μg/L, respectively) would not have clinical significance in patient sample analysis. The healthy population reference ranges for Cr and Co in blood are < 2.1 μg/L and <0.6 μg/L, respectively[29]. When analyzing MoM patient samples, clinically significant concentration differences in measuring hip prosthesis performance before and after the hip implant are considered to be 1 μg/L for Cr and 0.5 μg/L for Co [28]. Therefore, our Cr and Co concentration differences would have minimal impact on either of these concentration ranges. The data in this study indicated that there was no scientifically and clinically significant Cr and Co contamination from the limited set of stainless steel needles used.

Since most human blood samples from donors had Cr concentration below LOD, we decided to mimic the real-world collection in the lab (in-vitro), this time using spiked Cr and Co blood, 1.5 μg/L and 0.9 μg/L, respectively that is closer to 95th percentile concentrations in 2015-2016 NHANES[30]. Two blood tubes were collected consequently using same needle (200 blood tubes were collected using 100 needles). The concentration difference results for Cr and Co for 1st and 2nd blood tube have no clear trend (Table 2 and Figure SII). We performed paired sample t-test analysis to determine any statistically significant difference in Cr and Co concentrations between the 1st and 2nd blood tubes (Table 2). The paired sample t-test produced p-values above 0.05, indicating there is no statistically significant difference between Cr and Co concentrations in 1st and 2nd blood tubes collected in-vitro. We conclude that no Cr and Co leaching occurred into the blood sample using this particular set of stainless steel needles.

It is important to note, this conclusion does not apply to all blood collections. Lot screening results have shown the presence of contamination in some manufactured lots of needles. In

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the future, we plan to test multiple lots of needles and repeat these experiments using a lot of needles with known levels of Cr and Co contamination.

Long-term stability

We analyzed A and B aliquots stored at −70°C, −20°C, 4°C, and RT at 11 separate time intervals (E1=1 day; E2=1 week; E3=2 weeks; E4=4 weeks; E5=6 weeks; E6=2 months; E7=4 months; E8=6 months; E9=8 months; E10=10 months; E11=1 year) during 1 year period. Both Co and Cr analyte concentrations for all aliquots were within our established QAL (Table SV and Table SVI). At 4 and 10 months at −70°C, Cr concentration in B aliquots trended lower but was still within QAL, 10.82 and 10.69 μg/L, respectively (Table SVI). At 8 months, no A and B aliquots stored at RT could be analyzed. Fibrin micro clots had formed, making the matrix highly viscous; therefore, the samples could not be pipetted. Thus, we ended further analysis at this temperature.

To evaluate further the stability of Cr and Co human whole blood samples as a function of storage time and temperature, we conducted a statistical analysis of the data. First, we used a general linear regression model, including time and temperature as covariates to fit the data, with the measurement value as the outcome. Figure 2 presents model based Cr and Co mean estimate concentrations and the 95% confidence intervals as a function of temperature and time (concentrations can be seen in Table SVII and Table SVIII). No clear trend is present in Cr and Co concentrations in A and B pools as a function of temperature (Figure 2a and b). In Figure 2d, the Cr concentration in B pool at 1 year (E11) jumps slightly but is still within QAL.

We examined and compared Cr and Co recovery trends of bracketing QC with long-term stability A and B aliquots as a function of time over the one year to determine whether this jump is a sign of instability or the result of variations in experimental parameters (Figure SI and Figure 2). The trend in Cr and Co concentrations of bracketing QC and A and B long-term stability aliquots over the same time is similar (Figure SI and Figure 2). At E11, there were Cr concentration spikes in bracketing QC (Figure SI); therefore, we think outside factors, such as instrumental drift/fluctuations and variance in the sample preparation procedure unrelated to stability influenced the Cr spike at E11.

We evaluated the data using the equivalence test. Ratios of μ_{Test}/μ_{Ref} versus temperature and time are plotted in Figures 3 and 4, respectively. Numerical data can be found in Table SIX and Table SX. All p-values for both time and temperature were < 0.001 (Table SIX and Table SX). Since the p-values < 0.05, we rejected the null hypothesis (H_0) and accepted the alternative hypothesis (H_1: $0.8 \quad \mu_{Test}/\mu_{Ref} \quad 1.25$). Figure 3 shows equivalence test ratios of μ_{Test}/μ_{Ref} for Cr and Co results in A and B pools falling well within the equivalence boundaries, meaning Cr and Co concentrations in both pools at −20°C, 4°C, and RT were statistically equivalent to the Cr and Co concentrations at reference storage temperature −70°C. (Room temperature was only evaluated for the first 6 months.) Figure 4 displays equivalence test ratios of μ_{Test}/μ_{Ref} for Cr and Co concentration in A and B pools within the equivalence boundaries, meaning Cr and Co concentrations in both pools over one year were statistically equivalent to the Cr and Co concentrations at the reference storage of one day. The variation represented by error bars (95% confidence interval) in μ_{Test}/μ_{Ref} ratios at

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different temperatures (Figure 3) was slightly lower than the variation in μ_{Test}/μ_{Ref} ratios different times (Figure 4). Since μ_{Ref} is in the same analytical run for temperature but in a different analytical run for time than μ_{Test}, this slight variation was possibly due to instrumental drift/fluctuations, variance in sample preparation procedure (different analyst, reagents, and calibrators), or any other experimental parameters that can change daily. Overall, we concluded Cr and Co human blood samples are stable at temperatures −70°C, −20°C, and 4°C for one year and at RT for six months.

CONCLUSION

Based on the results from donor and in-vitro blood collection studies, we conclude there is no Cr and Co leaching from the limited sets of stainless steel needles used in these studies. In the future we plan to test multiple lots of needles to draw a more comprehensive conclusion on Cr and Co leaching from stainless steel needles. However, based on our lot screening data for needles, 8% (4 out of 49) of needle lots tested failed for Cr and Co. We cannot rule out the possibility of contamination of patient samples occurring if one of these lots with unacceptable levels of Cr and Co contamination are used. We conclude that the lot screening procedure is a critical step in controlling Cr and Co contamination and preventing incorrect Cr and Co concentration reporting in biomonitoring studies. A variety of other types of devices screened in our laboratory for Cr and Co had failures. These failures support the idea that all devices coming in contact with blood samples from sample collection to analysis and storage must be screened to avoid inaccurate results. We show that if blood samples are stored in prescreened cryovials, Cr and Co can be quantified correctly in human whole blood after being stored at temperatures of −70°C, −20°C, and 4°C for one year and at RT for six months. These are important findings and recommendations that should be considered in all biomonitoring studies that include the analysis of Cr and Co in whole blood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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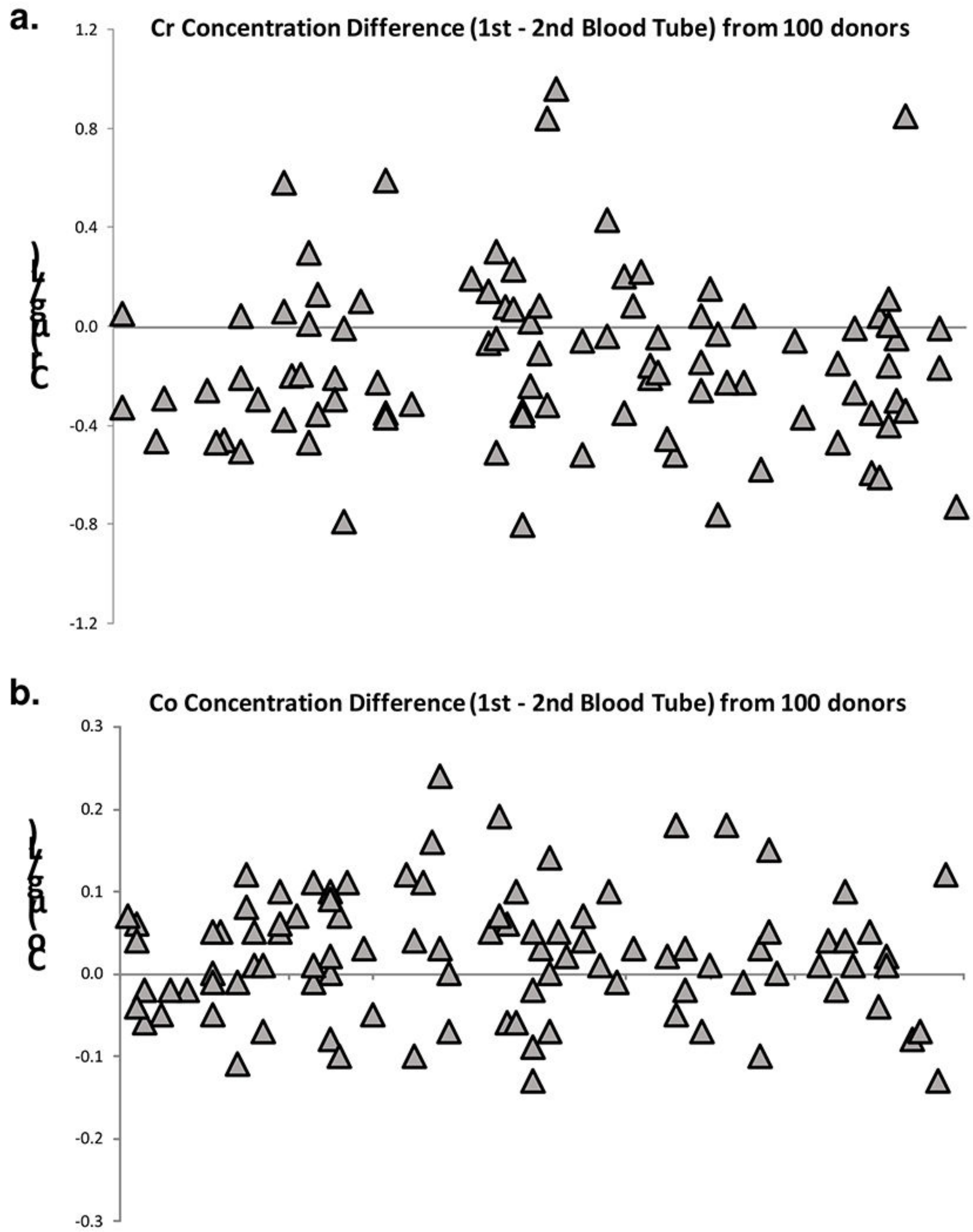

Figure 1.

The concentration difference in μg/L between 1st and 2nd blood tube (a) for Cr and (b) for Co collected from one hundred donors.

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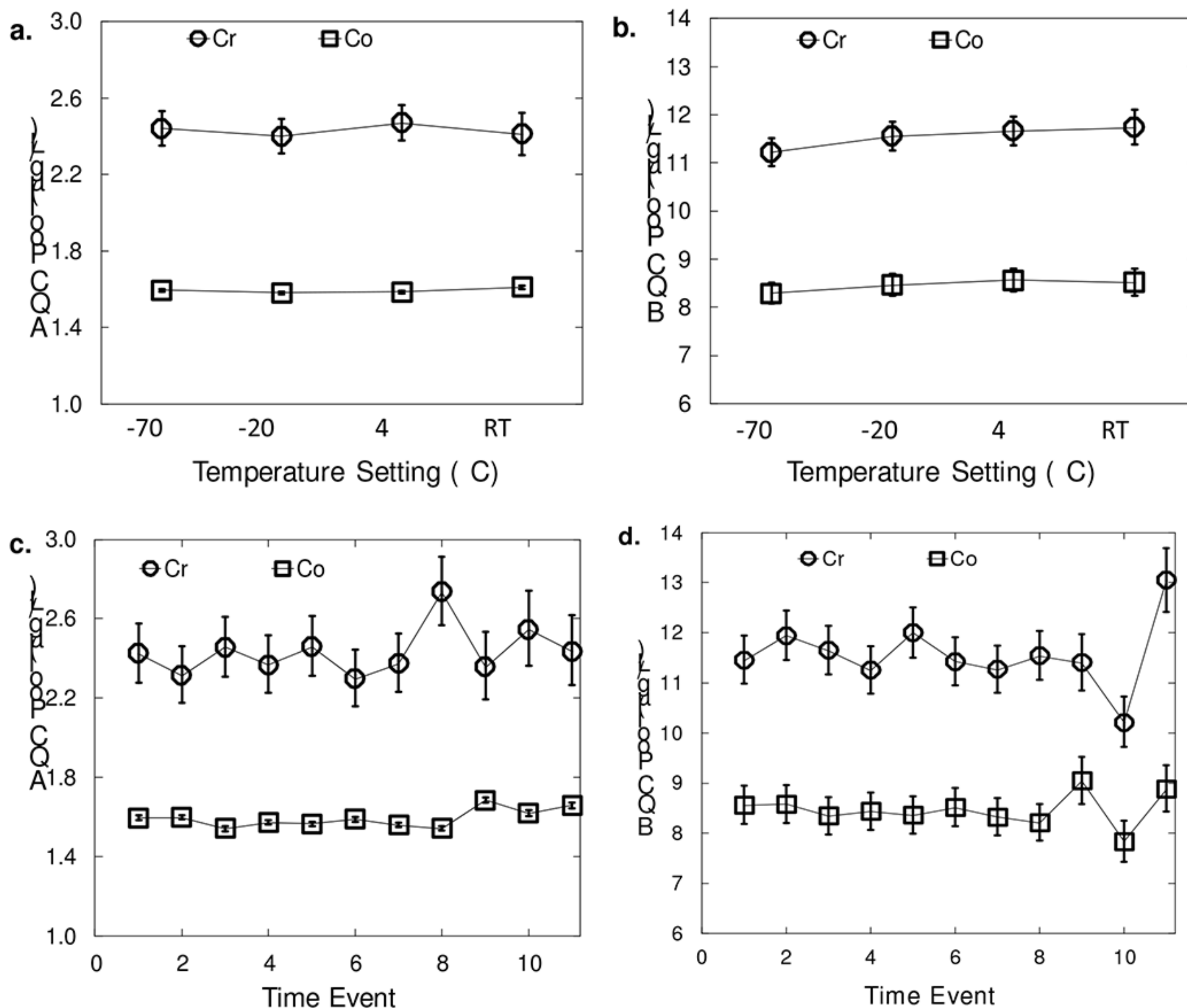

Figure 2.

General linear model based mean Cr and Co concentrations for low blood (A) and high blood (B) aliquots. Four different temperatures (−70°C, −20°C, 4°C, and RT) are shown in (a) for the A aliquots and in (b) for B aliquots. Eleven time events (E1=1 day; E2=1 week; E3=2 weeks; E4=4 weeks; E5=6 weeks; E6=2 months; E7=4 months; E8=6 months; E9=8 months; E10=10 months; E11=1year.) are shown in (c) for the A aliquots and (d) for the B aliquots. Each point is an average of results from three aliquots. Error bars represent the 95% confidence intervals.

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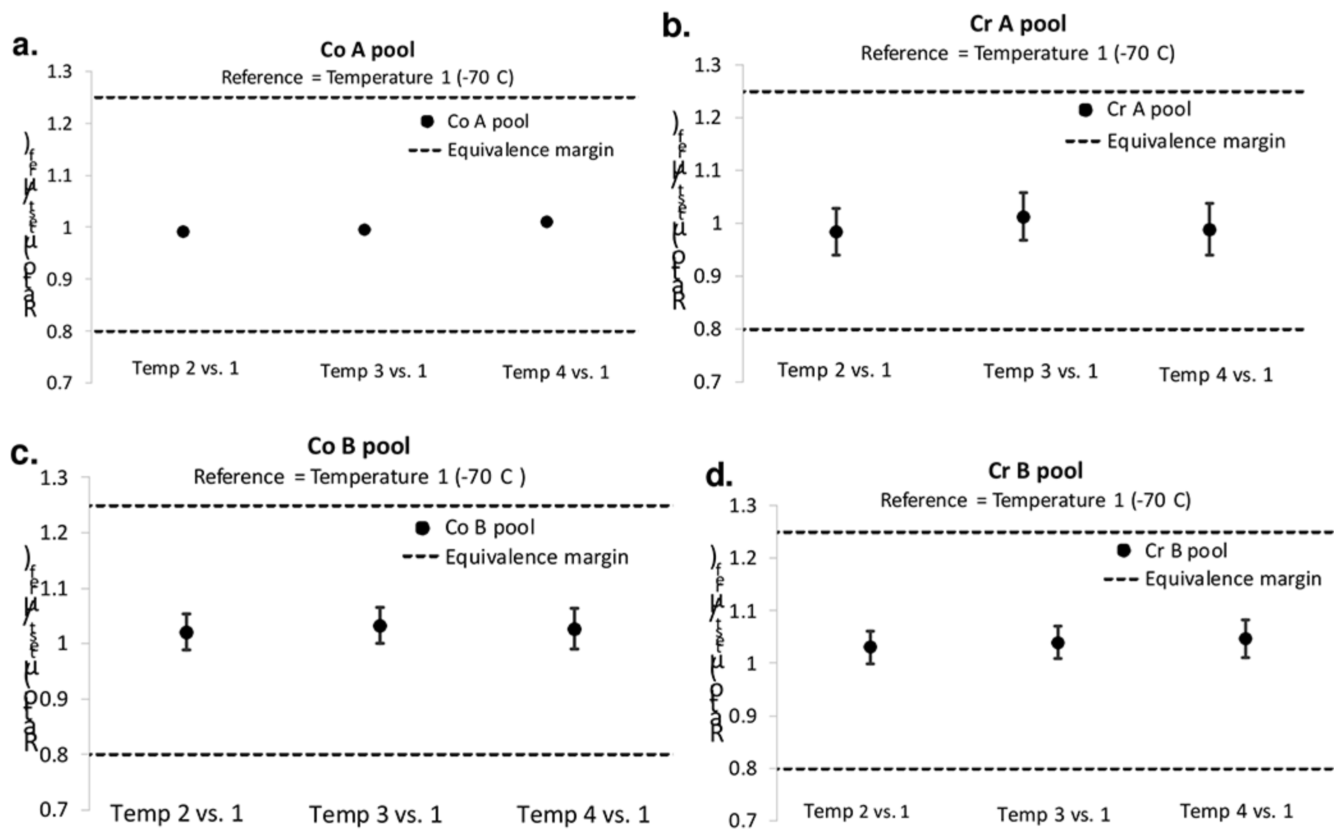

Figure 3.

Ratio of μ_{Test}/μ_{Ref} (equivalence test) for (a) Co in low pool (A), (b) Co in high pool (B), (c) Cr in low pool (A), (d) Cr in high pool (B). −70°C - Temp. 1; −20°C - Temp. 2; 4°C - Temp. 3; and RT - Temp. 4. Error bars are 95 % confidence interval.

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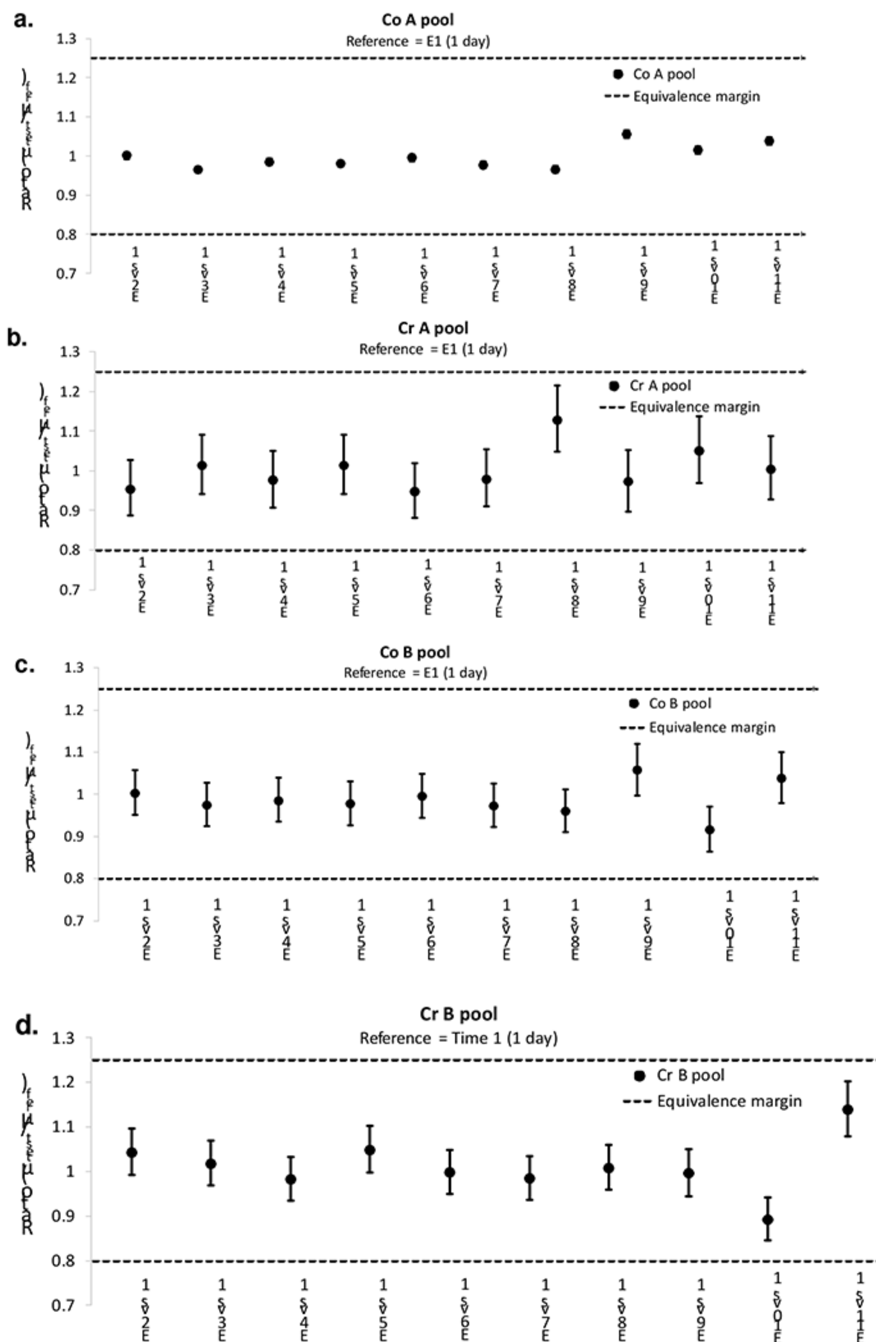

Figure 4.

Ratio of μ_{Test}/μ_{Ref} (equivalence test) for (a) Co in low pool (A), (b) Co in high pool (B), (c) Cr in low pool (A), (d) Cr in high pool (B). E1=1 day; E2=1 week; E3=2 weeks; E4=4 weeks; E5=6 weeks; E6=2 months; E7=4 months; E8=6 months; E9=8 months; E10=10 months; E11=1year. Error bars represent the 95% confidence intervals.

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Table 1:

Devices screened for Cr and Co contamination (November 2014 - January 2018). Maximum allowable contribution values are Cr - 0.09 μg/L and Co - 0.62 μg/L.

Device Type	Number of Lots Analyzed	Number of Lots Failed	Analyte Failures (lots)	% of Lots Screened that Fail
Needle	49	4	2 - Cr and 2-Co	8
Cryovial	16	0		0
Transfer pipette	6	1	Cr/Co	17
Vacutainer	58	12	10 - Co and 4 - Cr	21
Luer adapter	7	0		0
Microvette	1	1	Cr	100
Alcohol Pads	6	2	2 - Cr and 2-Co	33
Polypropylene tubes	11	2	1-Cr and 2-Co	18
Syringe	2	0		0

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Table 2:

1st and 2nd blood tube data analysis using paired sample t-test for Cr and Co (<0.05 statistically significant *). SD – standard deviation.

Analyte	N	Mean (1st - 2nd Blood Draw) (μg/L)	SD (μg/L)	P-value
2016 donors blood collection study				
Cr	100	−0.191	0.422	<0.0001
Co	100	0.024	0.076	0.002
2018 in-vitro study				
Cr	100	−0.010	0.110	0.4036
Co	100	0.001	0.040	0.783