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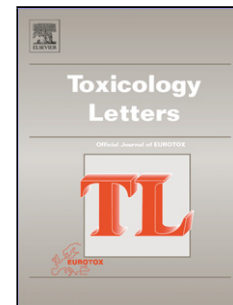


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Intra-erythrocyte chromium as an indicator of exposure to hexavalent chromium: an in vivo evaluation in intravenous administered rat.

Jérôme Devoy*, Frédéric Cosnier, Elodie Bonfanti, Guillaume Antoine, Hervé Nunge, Anne-Marie Lambert-Xolin, Marie-Josèphe Décret, Laurine Douteau, Mylène Lorcin, Sylvie Sébillaud, Stéphane Grossmann, Sylvie Michaux, Samuel Müller, Stéphane Viton, Carole Seidel and Laurent Gaté.

Institut National de Recherche et de Sécurité, Rue du Morvan, CS 60027, F-54519 Vandoeuvre-les-Nancy, France.

*Corresponding author (Jérôme Devoy)

Tel: +33-3 83 50 20 43; Fax: +33-3 83 50 20 96; E-mail address: jerome.devoy@inrs.fr

Highlights.

- Following an IV injection, this in-vivo study confirms rat urinary excretion peak at 6.3 hours and a nearly constant amount of intra-erythrocyte chromium for 2 days.
- A linear relationship exists between IV Cr(VI) dose and the Cr amount in erythrocytes up to 2 days.
- Unlike urinary chromium, Cr in erythrocytes may be a good biomarker of recent exposure to Cr(VI).

Abstract (150-200 words)

Hexavalent chromium (Cr(VI)) compounds are classified as carcinogenic to humans. Whereas chromium measurements in urine and plasma attest to the last few hours of total chromium exposure (all oxidation states of chromium), chromium in red blood cells (RBC) is attributable specifically to Cr(VI) exposure over the last few days.

Before recommending Cr in RBC (CrIE) as a biological indicator of Cr(VI) exposure, *in vivo* studies must be undertaken to assess its reliability. The present study examines the kinetics of Cr(VI) in rat after a single intravenous dose of ammonium dichromate. Chromium levels were measured in plasma, red blood cells and urine. The decay of the chromium concentration in plasma is one-phase-like (with half-life time of 0.55 day) but still measurable two days post injection. The excretion of urinary chromium peaks between five and six hours after injection and shows large variations. Intra-

erythrocyte chromium (CrIE) was very constant up to a minimum of 2 days and half-life time was estimated to 13.3 days. Finally, Cr(III) does not interfere with Cr(VI) incorporation in RBC. On the basis of our results, we conclude that, unlike urinary chromium, chromium levels in RBC are indicative of the amount of dichromate (Cr(VI)) in blood.

Abbreviations:

ACGIH-TLV-TWA: American Conference of Governmental Industrial Hygienists - Threshold Limit Value - Time Weighted Average

ANSES: The French Agency for Food, Environment and Occupational Health & Safety

BEI: Biological Exposure Indicator

CrA: Chromium in Atmosphere

CRI: Collision Reaction Interface

CrIE: Chromium in Erythrocyte (in RBC)

CrU: Urinary Chromium

ECHA: The European CHEMicals Agency

GEQUAS: The German External Quality Assessment Scheme (For Analyses in Biological Materials)

GF-AAS: Graphite Furnace Atomic Absorption Spectroscopy

IARC: The International Agency for Research on Cancer

ICP-MS: Inductively Coupled Plasma – Mass Spectrometry

NIOSH: National Institute for Occupational Safety and Health

OSHA: Occupational Safety and Health Administration

PBPK: Physiologically Based Pharmacokinetic

QMEQAS: Quebec Multielement External Quality Assessment Scheme

RBC: Red Blood Cells or erythrocytes

SVHC: Substance of Very High Concern

Keywords: Chromium, dichromate, erythrocyte, RBC, blood, rat.

1. Introduction

Chromium is a natural element found in rocks, animals, plants, and soils, where it exists in combination with other elements to form various compounds. Chromium is widely used in

manufacturing processes to make metal alloys such as stainless steel. It improves the resistance to oxidation. When combined with nickel or vanadium (and tungsten), it contributes to ductility and increases the resistance to high temperature (Weeks, 1932). Chromium can also be found in numerous consumer products such as wood treated with copper dichromate, leather tanned with chromic sulfate, stainless steel cookware, and metal-on-metal hip replacements (ATSDR, 2012). Chromium can exist in oxidation states ranging from -2 to +6. Of these, pure metal [Cr(0)], trivalent [Cr(III)] and hexavalent chromium [Cr(VI)] can all be encountered in the working environment. The major route of exposure is inhalation although the dermal route cannot be ruled out. The absorption of chromium depends on the speciation (or chemical species) and the solubility of the compound in the biological medium considered, as well as the particle size of the compound and its valency: in general, the trivalent derivatives are more poorly absorbed than hexavalent derivatives. Hexavalent derivatives include chromium trioxide, chromates and dichromates, and show variable solubility in water. Compounds such as chromium trioxide (CrO_3), alkali metal chromates (potassium chromate, sodium and lithium) are soluble. Alkaline earth metal salts (calcium chromate, strontium) are less soluble, whereas lead and zinc chromates are near insoluble (Koshi and Iwasaki, 1983).

Toxicokinetic data for Cr(III) and Cr(VI) are generally consistent with regard to absorption, distribution and excretion among different species (humans, dogs, rats, mice, rabbits, and hamsters). The soluble compounds of Cr(VI) are easily absorbed by the respiratory tract, are less well absorbed by the gastrointestinal tract and through the skin, are widely distributed in the body, and are eliminated in the urine and feces (Costa, 1997; Sun et al., 2015). Regardless of the route of exposure, Cr(VI) is reduced to Cr(III) by ascorbic acid or glutathione (Capellmann and Bolt, 1992; Zhitkovich, 2005). In rodents, a rapid detoxification of chromate by high concentrations of ascorbate make them more resistant to Cr(VI) toxicity and carcinogenicity in comparison to humans (Krawic et al., 2017). A PBPK model (O'Flaherty, 1996; O'Flaherty et al., 2001) has been developed based on rats; this model is quite sophisticated, but, as noted in the ATSDR toxicological profile, additional physiological and kinetic parameters from both humans and other animal species are still needed (ATSDR, 2012).

It is well known that Cr(VI) compounds are more toxic than Cr(III) compounds, being toxic by ingestion and inhalation and also presenting corrosive and sensitizing properties. Genotoxic effects are reported (Cieslak-Golonka, 1996; Nickens et al., 2010), as well as excess risks of cancer of the lung, nose and nasal sinuses. A reduction in sperm quality has been reported in subjects exposed to fumes containing Cr(VI) derivatives, but there is no evidence for impaired fertility or development (Kumar and Singh, 2015; Pizent et al., 2012). The IARC has classified Cr(VI) as a human carcinogen

(Group 1), in contrast with Cr(III) and metallic chromium, which are ‘not classifiable as to their carcinogenicity to humans’ (Group 3) (IARC, 1990, 2012).

In France, ANSES (the French Agency for Food, Environmental and Occupational Health & Safety) recommends an indicative Occupational Exposure Limit (OEL) (8-hour Time Weight Average) of 2 mg/m³ (total dust calculated as Cr) for Cr(0), inorganic Cr(II) compounds and inorganic Cr(III) compounds (ANSES, 2017) and a regulatory 8h-OEL of 0.001 mg Cr/m³ for Cr(VI) compounds (INRS, 2016). In comparison, in the United States, the Permissible Exposure Limits (PEL) established by OSHA (Occupational Safety and Health Administration) are 0.5 mg Cr/m³ for Cr(0), Cr(II) and Cr(III) and 0.005 mg Cr/m³ for Cr(VI). Recently, NIOSH, in considering all Cr(VI) compounds to be potential occupational carcinogens, has recommended a lowering of the 8-hour TWA to 0.0002 mg Cr/m³ (NIOSH, 2018).

While analysis of airborne chromium is well-documented from a methodological point of view and is therefore used for regulatory purposes, needs have been identified concerning the methods for determining biomarkers of exposure and effect (ATSDR, 2012). Exposure to higher than permissible exposure limits may result in increased chromium levels in blood, urine (CrU), exhaled air, hair, and nails. Elevated concentrations of chromium in blood and urine are considered to be the most reliable biomarkers of exposure. However, due to the rapid reduction of Cr(VI) to Cr(III) after absorption, blood and urinary chromium concentrations reflect the total amounts of chromium absorbed rather than the chromium absorbed after specific occupational exposure to Cr(VI).

The urine chromium assay, when used on samples taken at the end of the last shift of the working week, is thus considered a good indicator of not only the week’s exposure but also previous exposures to all forms of chromium. In contrast, samples taken at the beginning and end of the work shift allow evaluation of the exposure to soluble chromium during that particular work day. It should be noted that urinary chromium is subject to large intra- and interindividual variations. In addition, renal clearance of chromium is higher in chronically exposed workers than in workers only sporadically exposed. Even several months after the end of exposure, chromuria (CrU) may remain above the values of the general population (Goulart et al., 2005; Junaid et al., 2016). Some biological values have been established for Cr(VI) in occupationally exposed workers (welding or chrome plating activities). Thus, in the chrome plating sector, ANSES recommends a CrU of 2.5 µg/L (or 1.8 µg/g creatinine) at the end of the working week (ANSES, 2017). For exposure to welding fumes (soluble in water), the ACGIH recommends a CrU of 25 µg/L for the end of the work shift and working week (with a 10 µg/L increase during the work shift) (ACGIH, 2004). In the event of mixed exposure to Cr(VI) and Cr(III), considering the contribution of Cr(III) to CrU, urine measurements

should be interpreted in light of the respective levels of atmospheric concentrations for the various compounds of chromium. The level of CrU in the general adult population is equal to 0.65 $\mu\text{g/L}$ (or 0.54 $\mu\text{g/g}$ creatinine) (95th percentile) (ANSES, 2017).

Like CrU, with which it is well correlated, chromium measured in blood at the end of the last work shift of the working week will reflect long-term as well as recent exposure to chromium. Chromium in serum will only reflect the last two days of exposure (INRS, 2017).

In this study, the use of intra-erythrocyte chromium (CrIE) measurements for the characterization of Cr(VI) exposure is examined in order to assess whether CrIE would be specific for exposure to hexavalent chromium.

A previous *in vitro* study conducted on human blood samples confirmed that CrIE may be a good indicator of the internal dose resulting from exposure to Cr(VI) (Devoy et al., 2017). A linear relationship between CrIE and the amount of Cr(VI) added to blood was demonstrated, as was the robustness of CrIE with respect to several parameters (blood donors, blood reducing capacities, presence of Cr(III) in the medium).

The present work, conducted *in vivo* with rats following intravenous (iv) injection of Cr(VI) and/or Cr(III) solutions, aims to confirm our previous *in vitro* results and to compare the kinetics of Cr(VI) (and Cr(III)) incorporation and elimination in erythrocytes, plasma and urine. This study will provide additional data that will be of use for the improvement of PBPK models and for extrapolation across species for its use in risk assessment.

2. Experimental Method

2.1. Reagents and solutions

All chemicals used in the study were of analytical grade or higher. Nitric acid was used to prepare 0.1% HNO_3 (v/v) with ultrapure water (MilliQ, Millipore, Germany). All single element stock solutions (1000 mg/L) were provided by SCP Science and were certified for purity and concentration. From these stock solutions, a mixed working-standard solution with a concentration of 10 mg/L for each element was prepared by adding 1 mL of each stock solution to a 100 mL measuring flask, QSP 100 mL with 0.1% HNO_3 (v/v).

$(\text{NH}_4)_2\text{Cr}_2\text{O}_7$ (Sigma-Aldrich, 216615, 99%) and CrCl_3 (Sigma Aldrich, 27096) were chosen for Cr(VI) and for Cr(III) respectively. Cr(VI) and/or Cr(III) injected solutions were made from ammonium dichromate ($(\text{NH}_4)_2\text{Cr}_2\text{O}_7$) or chromium chloride (CrCl_3) dissolved in saline (0.9% NaCl), respectively.

2.2. Animals

While conducting the research described in this article, investigators adhered to the Guide for Care and Use of Laboratory Animals provided by the European Parliament and Council (Directive 2010/63/UE). Experimental procedures were approved by the local Ethical Committee and registered by the French Ministry for Research and Higher Education (APAFIS# 8913). The animal facilities are fully accredited (authorization n° D 54-547-10) by the French Ministry of Agriculture.

A total of 48 8-week-old male Sprague-Dawley OFA rats were used for the experiments in the current study. Eighteen of these were used to explore the kinetics of absorption and elimination of Cr(VI) in blood and in urine, whereas the other thirty were used to study the dose/effect relationship and Cr(III) incidence. The distribution of rats within the different groups is reported in Table 1.

Rats were purchased from Charles River laboratories (Domaine des Oncins, Saint-Germain-sur-l'Arbresle, France). Animals had a week of acclimatization prior to the experiments. Ten of them were catheterized at the level of the femoral vein. They were housed individually (for catheterized rats) or in pairs in polycarbonate cages (800 cm² x 18 cm height) with irradiated cellulose BCell8 bedding (ANIBED, Pontvallain, France). R04-10 irradiated pelleted food (SAFE, Augy, France) and filtered tap water were available ad libitum. Environmental enrichment was ensured by provision of a cardboard tunnel. Animals were maintained on a 12 h/12 h day/night cycle. Room temperature and relative humidity in the animal facility were maintained at 22±2 °C and 55±10 %, respectively.

2.3. Animal treatment and blood and urine collection

The rats were anesthetized with isoflurane prior to IV injection. As reported in Table 1, groups of treated rats received a single injection into the tail vein of 0.1 mL/kg body weight of Cr(VI) and/or Cr(III) solution. The solutions administered for the different groups contained either Cr(VI) (0.13, 1.31 or 2.62 mg of Cr(VI) per kilogram of rat), Cr(III) (1.31 mg Cr(III)/kg rat), or a Cr(VI)–Cr(III) mixture (each at 1.31 mg / kg rat). Control groups were administered saline solution only (0.9% NaCl).

Blood was collected in 300 µL heparinized tubes at different times (see table 1) from the tail vein of all rats except those used for the short-term (≤ 8 h) and mid-term (> 8 h and < 48 h) investigations of kinetics. For the latter two groups (short-term and mid-term groups), a femoral vein catheter was preferred in order to allow repeated collection over a short period of time. For ethical reasons and to avoid any measurement bias, the number of samples (= 7) collected in the short-term group allows not to exceed 10% of the total blood volume of rats. In addition, blood samples (collected in polypropylene heparinized tubes) were also collected from the tail vein, as well as at the

level of the abdominal aorta when animals were sacrificed after inducing deep anesthesia by intraperitoneal injection of a ketamine/xylazine mixture (75/10 mg/kg).

For urine collection, animals were housed in individual metabolic cage (type 3701M081 for rats over 300 g in weight; floor area of 450 cm²; Tecniplast, Lyon, France). Urine was collected over 48h for mid-term kinetics using either a static collection E-chiller that allows 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) or a refrigerated collection rack that allows one collection every 2 hours. Urine was collected over 24 h for long-term kinetics using a static collection E-chiller on post-exposure days 5, 30, 60 and 90. Urine samples were frozen after collection and stored at -20 °C, and thawed just before analysis.

2.4. Collection and preparation of biological samples

In order to eliminate any possible Cr contamination, all plasticware was soaked in 20% HNO₃ for 24 hours then rinsed three times with deionized distilled water before use. Between each sample collection, surgical instruments (scissors, scalpels and surgical clips) were rinsed with a 20% HNO₃ solution and then deionized distilled water.

Just after collection of blood (which took between 1 and 2 min), samples were centrifuged 5 min at 3000 g before removing the plasma. The process of chromium incorporation into red blood cells (RBC) is assumed to be stopped (or very strongly slowed down) from the beginning of the centrifugation step. RBC and plasma were sampled in tube and then stored in liquid nitrogen for later determination of the chromium concentration by GF-AAS (Graphite Furnace Atomic Absorption Spectroscopy) later on. .

Metabolism cages were used to contain experimental animals for the purpose of collecting urine (Harvard Apparatus, MA, USA; Braintree Scientific, MA, USA; Tecniplast, NJ, USA). These cages are specially engineered for separating faeces and urine into tubes outside the cage. The separation is immediate and complete with no urine washover and no risk of urine being contaminated by faeces.

Samples were collected over time in tubes in a refrigerated fraction collector positioned under the cages. This set-up allows interval urine collection in rats over periods as short as 2 hours to ensure adequate sample volumes.

2.5. Direct analysis of Cr using GF-AAS

Direct analyses of chromium in urine, plasma and RBC were performed using GF-AAS. A Varian AA280Z Atomic absorption spectrophotometer equipped with a Zeeman background

corrector was used for atomic absorption measurement of chromium at 357.9 nm and with a slit-width of 0.5 mm. A hollow cathode Cr lamp (SCP Science, 030-150-244) was operated at 3 mA. Uncoated graphite tube cuvettes were purchased from Schunk Kohlenstofftechnik (Germany). To reduce sample viscosity and dispense the sample more evenly in the graphite tube, aliquots of the RBC, plasma and urine samples were further diluted by a factor of 1:5 by adding 0.2% v/v HNO₃ and 1.0% v/v Triton X-100. A 30 µL volume of diluted sample was introduced to a graphite tube and atomized according to a set temperature program (Devoy et al., 2016). Peak areas in absorbance were used for quantitative analysis. Chromium calibration solutions were prepared in 0.2% v/v HNO₃ and 1.0% v/v Triton X-100. Method quantification limits for the original samples (before dilution or treatment) were 0.4 µg/L in RBC, urine, plasma and total blood.

The validation procedure was described in an previous paper (Devoy et al., 2013). The procedure provides estimates of the linearity limit, precision (intermediate precision, repeatability and reproducibility), accuracy and sensitivity for analysis of the element in question.

Chromium concentrations in plasma and in RBC are expressed (in mg/L) by volume of plasma and by volume of blood, respectively.

2.6. Mathematical modeling and statistics

All statistical analyses, linear regressions and curve modeling were performed using GraphPad Prism Software (Version 6.00 for Windows) (La Jolla California USA, www.graphpad.com).

Chromium concentration profiles (in urine, plasma or erythrocyte) were fitted with the common one-phase decay or one-phase association equations:

- One-phase decay equation: $Y = (Y_0 - P) \cdot \exp(-Kt) + P$
- One-phase association equation: $Y = Y_0 + (P - Y_0) \cdot (1 - \exp(-Kt))$

where P is the Y value at infinite times (Plateau), expressed in the same units as Y; Y₀ is the Y value when X (time) is zero, expressed in the same units as Y; K is the rate constant, expressed in reciprocal of the X axis time units. If X is in minutes, then K is expressed in inverse minutes; half-life is in the time units of the X axis. It is computed as $\ln(2)/K$.

One-way analysis of variance (ANOVA) was used to analyze the metabolic activity of the different groups. Post hoc tests were performed using the Tukey method. Differences between groups were considered to be significant at a P value of <0.05. Two-way ANOVA was used to take into consideration the post-exposure time factor (in addition to the group factor).

3. Results

3.1. General health

The exposure conditions (single-compound exposure or binary exposure) did not affect weight-gain in animals (average animal weight = 329 ± 23 g and 537 ± 33 g at 2 and 90 days post-injection, respectively). Exposure to Cr(VI) (even at doses of 2.6 mg Cr(VI) / kg rat) did not induce any visible signs of an acute toxicity. Average daily diuresis for all animals was normal: 12.5 ± 8.5 mL. Urinary creatinine was stable within the groups (1.10 ± 0.38 g/L). Finally, RBC viability was not affected by the different treatments ($85.9 \pm 5.2\%$ on average).

3.2. Kinetics of absorption and elimination of Cr(VI) in blood and in urine

CrU measured over time after a single injection of 2.6 mg Cr(VI) / kg rat showed very high inter-individual variability. However, as shown in Figure 1, the mean maximum urinary concentration ($C_{max} = 57.7$ mg/L) was obtained 0.26 days ($t_{max} \approx 6.3$ hours) after injection with a width at half-height of 4.3 hours. The excretion rate peaked at 26.2 mg/L/h. CrU then decreased rapidly along a one-phase decay curve (fitted between t_{max} and day 90) characterized by the equation $[CrU] = (Y_0 - P) \cdot \exp(-K_e \cdot t) + P$, with $Y_0 = 1624 \pm 897$ mg/L, plateau $P = 4.12 \pm 0.9$ mg/L and elimination rate constant $K_e = 13.08 \pm 2.05$ day⁻¹. Goodness of fit was demonstrated by $R^2 = 0.62$. At days 60 and 90, CrU levels were identical (around 80 µg/L) in both injected and control animals. In terms of excreted amount, most of the Cr eliminated in urine, representing 26.7 % of injected dose, occurs in the first 48h (Figure 2).

In plasma, the decrease in Cr concentration (CrP) (over time $t_0 \rightarrow d_{90}$) can be described by first-order elimination kinetics, with $CrP(0) = 6.57 \pm 0.22$ mg/L and the elimination rate constant $K_{eP} = 1.55 \pm 0.13$. After 48h, only 10% of the initial concentration remained in plasma (Figure 3) (less than 1% after 4 days). Very low inter-individual variability is observed at each collection time.

Inter-individual variability is slightly higher with CrIE, with a mean relative standard deviation below 10% during the first 2 days. The incorporation of chromium in erythrocyte was very rapid (less than one hour). The maximum concentration of CrIE (5.1 ± 1.2 mg/L, representing 0.24 % of the injected dose) was measured 5 minutes post-injection. An equilibrium phase was observed in the first hours (2-6 hours after injection) following injection, leading to a slight decrease in concentration before reaching a plateau lasting 2 days (around 4.3 mg/L) (Figure 3). Overall, between the injection and day 90, [CrIE] follows a one-phase decay curve with a $t_{1/2}$ of around 14 days and background level reached at day 60 (Figure 4).

3.3. Dose–effect relations and incidence of Cr(III)

The amount of Cr excreted in the urine of control animals was $0.022 \pm 0.014 \mu\text{g/h}$, or approximately $50 \mu\text{g/L}$. The amount of urinary Cr excreted in the 48 hours following injection increased proportionally with the injected dose (Cr(VI) and/or Cr(III)) regardless of the collection period considered (0 h – 8 h, 8 h – 24 h or 24 h – 48 h) (Table 2). As a consequence, the CrU levels measured at different time-points were also proportional to the injected doses of Cr(VI) (Fig. 5A). However, as demonstrated by the significant differences ($p=0.002$) in slope (ranging from 2.06 to 18.66) for the different linear relationships, excretion depended on time. As mentioned earlier, most of the excretion occurred during the first 8 h, representing 39% to 61% (depending on the treatment) of the total over 48 h (itself corresponding to 18.7 to 32.0% of the initial injected dose). Over the first 8 h, the amounts excreted in urine in the “Cr(III) = 1.3 mg/kg” group and “Cr(VI) = 1.3 mg/kg” group were comparable ($38.89 \mu\text{g}$ and $41.47 \mu\text{g}$). Moreover, over the same first 8 h period, the amounts excreted in urine by the “Cr(VI) = 2.6 mg/kg” and “Cr(III) + Cr(VI) = 1.3 + 1.3 mg/kg” groups are comparable ($141.89 \mu\text{g}$ and $159.68 \mu\text{g}$) and were proportional to injected dose (Table 2). During the two subsequent collection periods (8 h - 24 h and 24 h – 48 h), Cr(III) groups (1.3 mg/kg or the mixture with Cr(VI)) excreted less (31 and 35%, respectively) than the corresponding Cr(VI) groups.

Concentrations of Cr(VI) found in RBC and plasma of controls were largely below the LOQ of the method ($= 2 \mu\text{g/L}$) (Table 3). After the equilibration period (under 5 hours), CrIE increased proportionally with the injected doses of Cr(VI) (Figure 5B). CrIE then remained stable until at least 48 hours post injection, as stated earlier and as demonstrated by the absence of statistical differences in terms of the slopes (ranging from 1.675 to 1.988 with a pooled slope equal to 1.80, $p=0.69$) and elevations (pooled intercept equal to 0.149, $p=0.53$) of the linear relationships obtained at different times post injection (Figure 5A). In contrast, treatment with Cr(III) (at 1.3 mg/kg bw) induced no observable increase in CrIE (almost 200 times less than for Cr(VI) at 1.3 mg/kg bw). In addition, the two-way ANOVA results (group, post-exposure time with time greater than 1 hour) show a slight incidence of Cr(III) on the integration of Cr(VI) in RBC ($p = 0.02$): the CrIE in the $2 \times 1.3 \text{ mg/kg}$ (Cr(VI) + Cr(III)) group is on average 11% lower than it is in the 1.3 mg/kg Cr(VI) group (Fig. 6).

In the plasma compartment, at equivalent mass injected doses, the CrP measured in the 1.3 mg/kg Cr(III) group is statistically higher than the CrP measured in the 1.3 mg/kg Cr(VI) group (probably due to the partial integration of Cr in RBC in the latter) (Table 3). CrP resulting from injection of the mixed solution was equal to the sum of the two contributions (Cr(III) and Cr(VI)) ($p=0.13$). For Cr(VI) groups, assuming a volume ratio of $\frac{1}{2}$ between RBC and plasma, nearly 50% of

Cr reached the RBC after the injection. For each species of Cr, the decrease in CrP was rapid, and the kinetics of elimination do not appear to differ between the two species ($t_{1/2}$ of less than 10 hours).

4. Discussion

The PBPK model proposed by O'Flaherty in 1996 (O'Flaherty, 1996) was based on data reported in various intravenous, oral and intratracheal rat studies. The adaptation of the model for humans described by O'Flaherty in 2001 (O'Flaherty et al., 2001) was based on data obtained from subjects that had ingested doses of Cr(VI) or Cr(III). Ingestion has been a particularly well-studied route of administration, especially for purposes of model development (Kirman et al., 2013; Proctor et al., 2012). Species-related differences in Cr pharmacokinetics have been demonstrated; for example, mice and rats have been found to display different distributions of Cr via non-IV routes of exposures and different capacity to sequester Cr(VI) in erythrocytes (Kargacin et al., 1993). Studies directly examining rodents and humans differences are limited (ATSDR, 2012). The data provided in the present study should bring new data for comparison and enable consolidation of these models. Particular focus was given to urinary and blood (especially RBC) measurements because of their uses in the context of biomonitoring.

To compare the performance of Cr in erythrocytes (CrIE) and in urine (CrU) as potential biomarkers of exposure of Cr(VI), rats were intravenously injected with Cr(VI) and/or Cr(III) solutions in order to study the kinetics of incorporation and elimination. The intravenous route was chosen in order to compare the concentrations in the different matrices and associated biomarkers. While this administration route would not be relevant in an occupational exposure context, given that Cr(VI) is more readily adsorbed from the lungs into the bloodstream or by mucociliary clearance than Cr(III) (53-85 % and 5-30 %, respectively) (ATSDR, 2012), it was chosen because of its ability to overcome the differences in the adsorption of Cr(VI) and Cr(III) and thus the results for the two Cr species can be compared more directly at equivalent blood level. However, in physiological routes of exposure, Cr(III) can enter the bloodstream only in the form of complexes with some organic ligands (citrate, amino acids, ...). While it is convenient to use inorganic Cr(III) salt, as done here, behavior of organic Cr(III) complexes with respect to entry into RBC can be different (Zhitkovich, 2005).

Integrating allometric adjustments, the IV doses were equivalent to approximately 1, 10 and 20 times the cumulated amount that would enter the bloodstream of a human exposed to the 8h-OEL of Cr(VI) ($0.001 \text{ mg} / \text{m}^3$) over their entire working life (39 hours per week, 47 weeks per year for 40 years), assuming a human respiratory minute volume (of $7.5 \text{ L}/\text{min}$), a lung deposition fraction of 20%, 70% absorption in blood, and human and rat total alveolar surface areas of 75 and 0.57 m^2 , respectively.

These doses do not trigger clinical signs of toxicity (Fairhurst and Minty, 1989). In addition, the amounts injected into the blood were such that they did not exceed the reduction capacity and they had at worst a very limited impact on the hematological parameters, the viability of RBC being unaffected (Devoy et al., 2016; Ray, 2016).

In the present conditions, in agreement with literature data and similar to results obtained for humans, the half-life for urinary excretion was found to be short, between 3 and 10 hours based on an open, one-compartment kinetic model (Kiilunen et al., 1983); it is likely that the other excretion peaks for two other biological half-lives were not visible (ATSDR, 2012). Similarly, the amounts in percent (around 30%) eliminated in total over the 48 hours following injection are in agreement with those observed after IV-administration of chromium (in potassium dichromate form) in male Wistar rats (Sayato, 1980). The fast elimination property is used to monitor the exposure of workers to chromium and to set biological limit values. Thus, urinary chromium measurements made at the end of the last work shift of the working week could be a good indicator of recent and long-term occupational exposure to all forms of chromium (including hexavalent forms) (CrU = 25 µg/L for ACGIH and 2.5 µg/L for ANSES). In our exposure conditions, the level of CrU in controls (approximately 50 µg/L) exceed twice the value recommended by ACGIH. This could be explained by the composition of the food used to feed the animals. Wistar rats fed by a casein-based semi-purified diet supplemented by only 1 µg Cr/g (as CrCl₃) excreted 0.41 µg Cr/day in urine (Yoshida et al., 2010). This somewhat limits extrapolation of our data between animals and humans, but it also illustrates an issue of diet that also exists in humans.

Taking into consideration the value for the general population (Human CrU = 0.65 µg/L) (ANSES, 2017), the ratio between the value for the general population compared to proposed biological values (~ ×4 for ANSES biological value or ~ ×40 for BEI ACGIH) and the level of CrU in control rats (approximately 50 µg/L), the equivalent biological values in rat should be 0.2 mg/L for ANSES or 2 mg/L for ACGIH. In urine, a level of 0.2 mg/L can be reached 5 h or 48 h post exposure with injection of only a few µg whereas a level of 2 mg/L is obtained at 5 h and 48 h post exposure with around 30 µg and 800 µg, respectively (Figure 5A). This illustrates the difficulties in evaluating the level of exposure from CrU measurements even in the controlled and ideal conditions of our laboratory experiments. In an occupational context, the probability of detecting/identifying an acute exposure that took place early in the week via a measurement of urinary chromium made at the end of the last working day of the working week would likely be even lower. To this can be added the effects of the physical form of atmospheric chromium, its solubility and the chemical species of chromium (speciation). As already mentioned previously (Cikrt and Bencko, 1979) and also shown here, there is almost no difference between urinary Cr(VI) and Cr(III) excretion profiles. Moreover,

CrU can also be affected by renal function, diabetes (Bukowski et al., 1991) and a lack of exercise, smoking status (Kalliomäki et al., 1981; Stridsklev et al., 1993; Stridsklev et al., 2004) and/or the beer consumption habits of volunteers. Furthermore, renal clearance of chromium is also higher in chronically exposed workers than in workers exposed only occasionally. Ultimately, all these factors contribute to large intra- and inter-individual variations.

In 1993, Gao et al. (Gao et al., 1993) monitored the total chromium present in rat fluids following intratracheal administration of soluble trivalent and hexavalent chromium compounds. Wistar rats exhibited increased chromium concentrations in whole blood, plasma and urine up to 72 h post exposure. The ratio of whole blood chromium to plasma chromium concentrations differed significantly for Cr(VI) and Cr(III) treatments and the authors therefore recommended use of both blood and plasma chromium assays to assess chromium exposure. Following IV administration, the CrIE/CrP ratio also varies significantly depending on the nature of chromium speciation (Cr(VI) or Cr(III)), however, this parameter would not appear to be convenient for discriminating between exposure to one or other form of Cr, especially given the rapid evolution of CrP over time (90 % disappearance in 48h), as mentioned by Sayato et al. (Sayato, 1980).

Thus, CrP could be considered for evaluating recent exposure to Cr(III) or Cr(VI), whereas CrIE would allow evaluation of Cr(VI) exposure over a long period. Under our IV conditions, the fact that CrIE background level is reached after 60 days is simply linked to the life expectancy of rat RBC, (61.0 ± 1.3 days for the Sprague-Dawley rat (Derelanko, 1987)). Once trapped in RBC, Cr(VI) is confined there until the death of the RBC. Given that the life expectancy of human erythrocytes is 120 days, we can therefore estimate that the Cr measured in human RBC corresponds to an integrated period of time that is almost double that of rat RBC with an initial plateau of up to 5 days. Some field studies have already evaluated the possibility of using CrIE as specific marker for chromium (VI) exposure (Goldoni et al., 2010; Lukanova et al., 1996; Zhang et al., 2006; Zhang et al., 2012; Zhang et al., 2011), although some authors criticized the lack of sensitivity of CrIE (Kortenkamp, 1997). The conditions under which these investigations were carried out are not comparable and it is not therefore possible to draw any definitive conclusions about the relevance of this marker and its performance compared to other potential biomarkers (Cr levels in urine and lymphocytes). This also raises questions about the validation protocol and the relevance of the validation of a biological indicator with the other data collected (atmospheric measurements, surface swipes, skin swipes, urinary data, etc.).

Regarding the question of sensitivity, only a few studies have reported CrIE concentrations in the general (control) population; the levels measured exceed $1 \mu\text{g/L}$ (2.50 ± 1.53 (Lukanova et al., 1996), 3.21 ± 2.20 (Zhang et al., 2006), $2.64 \mu\text{g/L}$ (Qu et al., 2008) and $1.54 \mu\text{g/L}$ (Zhang et al., 2011)).

Considering only inhalation exposure at the currently recommended OELs (1 and 0.2 $\mu\text{g}/\text{m}^3$ in France and in the US, respectively), it is clear that the CrIE level would stay close to the endogenous level. According to the Biotox INRS database (INRS, 2006), during exposure to alkaline chromates (Cr (VI)) for CrO_3 airborne concentrations in the range of 30 - 50 $\mu\text{g}/\text{m}^3$, corresponding CrIE concentrations at the end of the work shift were between 9 and 17 $\mu\text{g}/\text{L}$ of whole blood at the end of the work shift respectively (EKA values, not applicable for welders). Unlike CrU, CrIE is unaffected by a lack of exercise, beer consumption, diabetes or smoking status (Bukowski et al., 1991); any rise in CrIE concentration should therefore be questioned and should lead to exploration of the other routes of exposure (ingestion or cutaneous).

In our experimental conditions, CrIE is undoubtedly a much better indicator than CrU. Nevertheless, work is still necessary to fully validate its use for biomonitoring purpose in an occupational health and safety context. In addition, the association between DNA damage in peripheral lymphocytes and chromium level in erythrocytes in electroplating workers demonstrated by Zhang et al. (Zhang et al., 2011) suggests that additional studies of this marker are warranted.

5. Conclusions

This *in vivo* study confirms that hexavalent chromium enters rat RBC and that the amount of chromium in the RBC is monotonically linked to the amount of hexavalent chromium present in the blood following exposure. Our study has also highlighted that quantitative amounts of hexavalent chromium, unlike Cr(III), enter the RBC and remain there for several days.

To assess the selectivity of this indicator, we also examined the influence of trivalent chromium on the incorporation of Cr(VI).

The kinetics of chromium in plasma, in urine and in RBC were compared. Two days after IV administration of Cr(VI), less than 20% of the initial dose is measurable in plasma and “blank equivalent chromium” concentrations are detectable in urine, whereas the chromium in RBC is still over 80% of the initial level.

The results confirm that CrIE could be used as an indicator of the internal dose resulting from recent exposure to hexavalent chromium (during the preceding 2-5 days for rats and possibly longer for humans).

The final step will be to firmly link real hexavalent chromium exposure to measured RBC chromium. More *in vivo* data needs to be collected to confirm the relationship and allow CrIE to be proposed as a biological indicator of hexavalent chromium exposure. Future perspectives could include studying the kinetics of intra-erythrocyte chromium after inhalation in rats, which may reveal

a correlation between chromium levels in exhaled breath condensate and in RBCs, as suggested by Goldoni et al. (Goldoni et al., 2010) in their field study.

Conflict of interest

The authors declare that there are no conflicts of interest.

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ACCEPTED MANUSCRIPT

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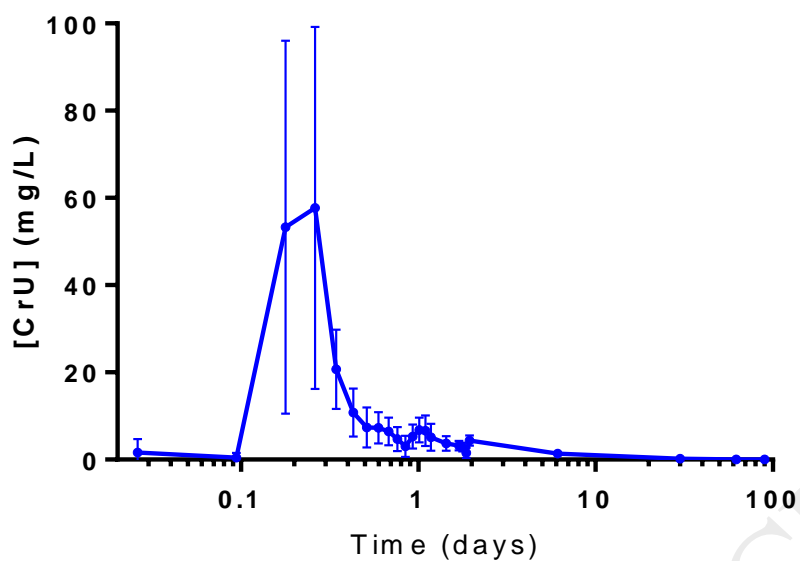


Fig. 1. Urinary chromium (CrU) concentration as a function of time. Chromium profiles after intravenous injection of a single dose of ammonium dichromate (2.6 mg / kg body weight) in rats. $C_{\max} = 57.72$ mg/L at $t_{\max} = 0.261$ day (6.26 h). $N = 8$ rats.

Best fit equation for CrU elimination between 0.3 and 90 days: $Y = (Y_0 - P) \cdot \exp(-Kt) + P$, with Plateau $P = 4.12$ mg/L; $Y_0 = 1624$ mg/L and $K = 13.08$ day⁻¹.

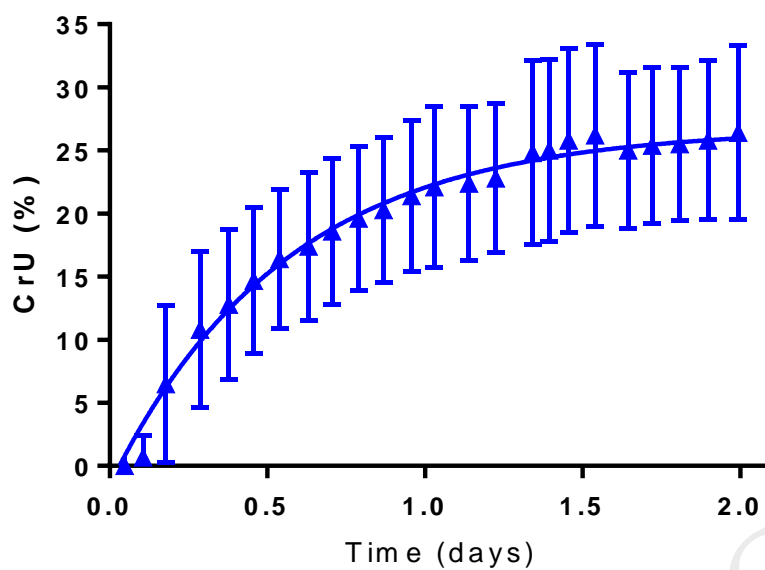


Fig. 2. Urinary chromium profile after intravenous injection of a single dose of ammonium dichromate (2.6 mg / kg body weight) in rats. Cumulated relative dose of chromium as a function of time.

Best fit equation for CrU : $Y = Y_0 + (P - Y_0) * (1 - \exp(-Kt))$, with Plateau $P = 26.74\%$; $Y_0 = -1.511\%$ and $K = 1.797 \text{ day}^{-1}$.

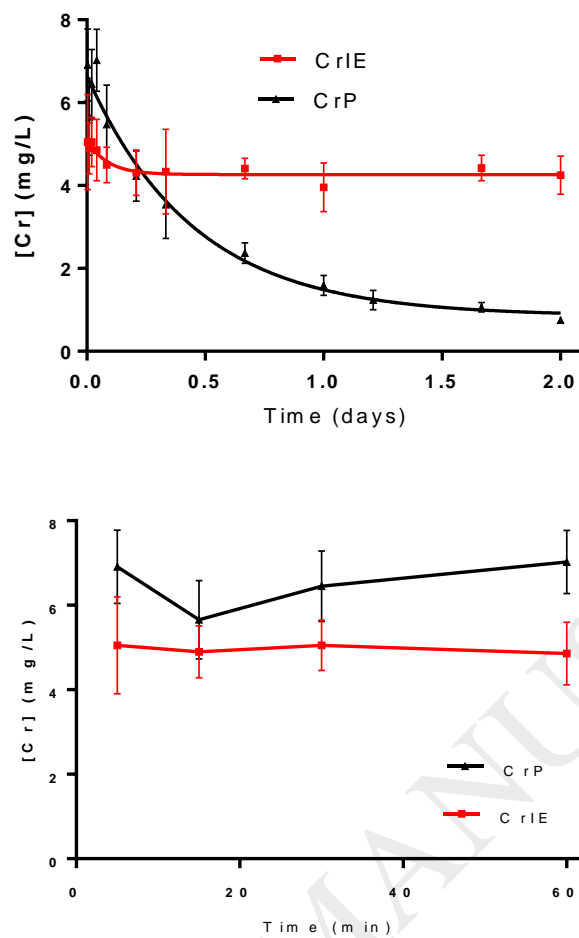


Fig. 3. Chromium in red blood cells (CrIE) and in plasma (CrP) as a function of time. Chromium profiles after intravenous injection of a single dose of ammonium dichromate (2.6 mg / kg body weight) in rats. N = 8 rats.

Best fit equation for CrIE: $Y = (Y_0 - P) \cdot \exp(-Kt) + P$, with Plateau $P = 4.265$ mg/L; $Y_0 = 5.101$ mg/L and $K = 11.55$ day⁻¹. Best fit equation for CrP: $Y = (Y_0 - P) \cdot \exp(-Kt) + P$, with Plateau $P = 0.079$ mg/L; $Y_0 = 6.567$ mg/L and $K = 1.552$ day⁻¹.

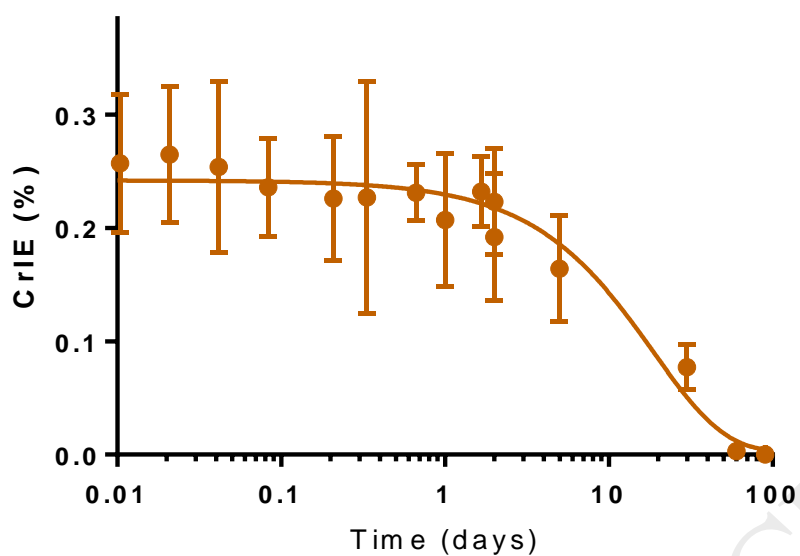


Fig. 4. Chromium fraction in red blood cells (in 300 μ L of blood) as a function of time. Chromium profile after intravenous injection of a single dose of ammonium dichromate (2.6 mg / kg body weight) in rats. $t_{1/2}$ = 13.3 days.

Best fit equation for CrIE: $Y = (Y_0 - P) \cdot \exp(-Kt) + P$, with Plateau $P = 0.0024$ %; $Y_0 = 0.2421$ % and $K = 0.053$ day $^{-1}$.

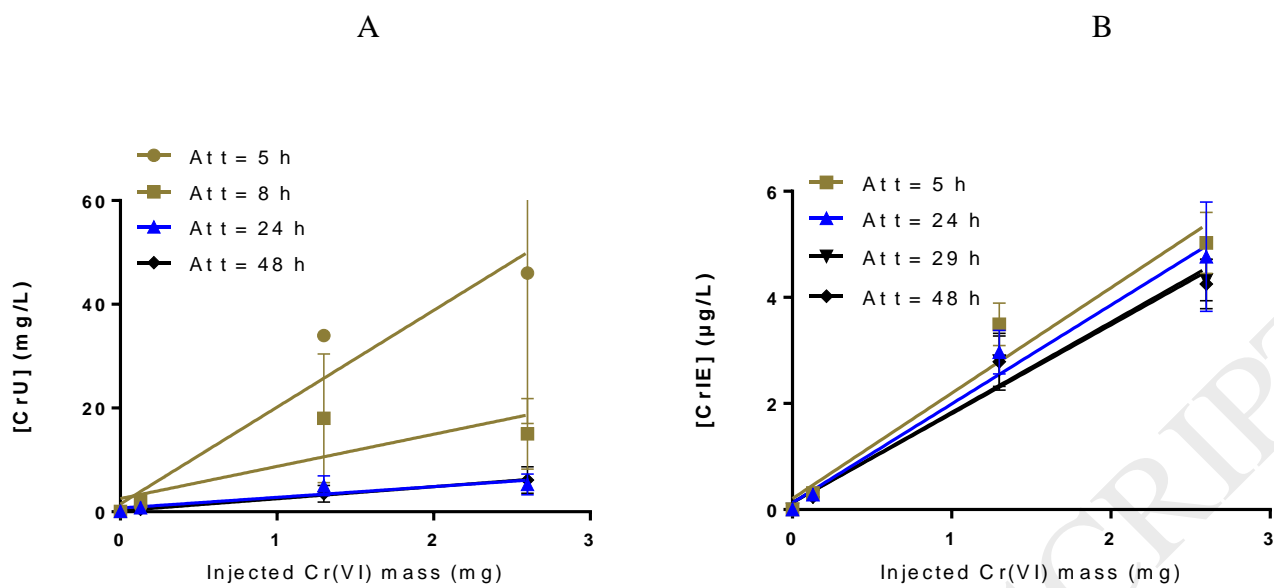


Fig. 5. Linear relationship between Cr(VI) injected mass at different times after a single dose injection and (A) urinary chromium or (B) chromium in red blood cells. N = 6 rats / dose.

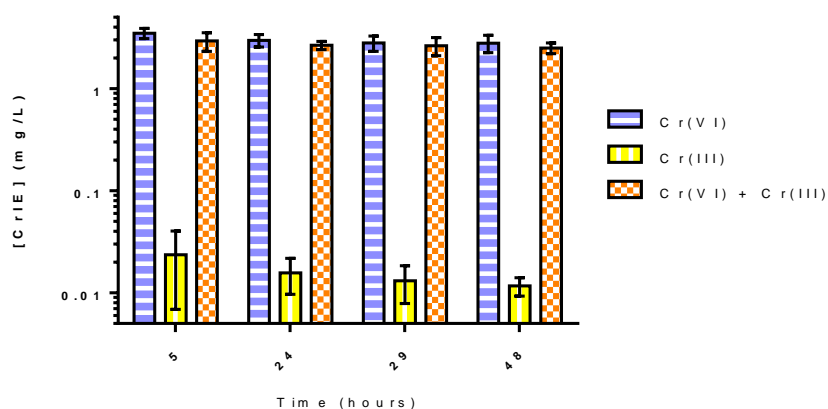


Fig. 6. Influence of Cr(III) on incorporation of hexavalent chromium in red blood cells as a function of time. “Cr(VI)” is equivalent to a single dose of 1mL/kg of rat at $[(\text{NH}_4)_2\text{Cr}_2\text{O}_7] = 3.12 \text{ g/L}$, i.e. 1.3 mg of Cr(VI)/rat. “Cr(III)” is equivalent to a single dose of 1mL/kg of rat at $[\text{CrCl}_3] = 3.93 \text{ g/L}$, i.e. 1.3 mg of Cr(III)/rat. “Cr(VI) + Cr(III)” is equivalent to a single dose of 1mL/kg of rat at $[(\text{NH}_4)_2\text{Cr}_2\text{O}_7] = 3.12 \text{ g/L} + [\text{CrCl}_3] = 3.93 \text{ g/L}$, i.e. 1.3 mg of Cr(VI) and 1.3 mg of Cr(III) /rat. N = 5 rats / group.

Table 1. Characteristics of the different groups of rats and summary of the treatments undergone (injection, collection of blood and urine)

| Group | Uses | Injection | Number of animals | Blood collection time points | Urine collection time points |
|-------|--|---|-------------------|---|--|
| #1 | Kinetics Short term | 2.6 mg Cr(VI) / kg rat | 6* | 5 min, 15 min, 30 min, 1 h, 2 h, 5 h and 8 h | |
| #2 | Kinetics Mid term | 2.6 mg Cr(VI) / kg rat | 4* | 16 h, 24 h, 40 h and 48 h | Every 2 h for 48 h |
| #3 | Kinetics Long term | 2.6 mg Cr(VI) / kg rat | 4 | 48 h, d5, d30, d60 and d90 | Every 2 h for 48 h and for 24 h on d5, d30, d60 and d90 |
| #4 | Kinetics Controls | Saline solution | 4 | 24 h, 48 h, d5, d30 and d90 | Every 2 h for 48 h |
| #5 | Dose/effect | 1.3 mg Cr(VI) / kg rat | 5 | 1 h ,5 h, 24 h, 29 h and 48 h | Every 2 h for 48 h (with 2 rats) or 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) with 3 rats |
| #6 | Dose/effect | 0.13 mg Cr(VI) / kg rat | 5 | 1 h ,5 h, 24 h, 29 h and 48 h | Every 2 h for 48 h (with 2 rats) or 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) with 3 rats |
| #7 | Cr(III) incidence | 1.3 mg Cr(III) / kg rat | 5 | 1 h ,5 h, 24 h, 29 h and 48 h | Every 2 h for 48 h (with 2 rats) or 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) with 3 rats |
| #8 | Cr(III) incidence | 1.3 mg Cr(III) / kg rat + 1.3 mg Cr(VI) / kg rat | 5 | 1 h ,5 h, 24 h, 29 h and 48 h | Every 2 h for 48 h (with 2 rats) or 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) with 3 rats |
| #9 | Dose/effect & supplements for kinetics | 2.6 mg Cr(VI) / kg rat | 5 | 1 h ,5 h, 24 h, 29 h and 48 h | Every 2 h for 48 h (with 2 rats) or 3 collection periods (first 8 h, 8 h – 24 h or 24 h – 48 h) with 3 rats |

* Rats were catheterized at the level of the femoral vein

Table 2. Amounts of chromium excreted in urine over time according to the type of intravenous treatment.

| | Injected dose (ID) (mg Cr/kg bw) | Amount of Cr (μ g) excreted in urine* | | | % of ID excreted in urine at 48h |
|-------------------------|----------------------------------|--|-------------------|-------------------|----------------------------------|
| | | 0h – 8h | 8h - 24h | 24h – 48h | |
| Control | Saline | 0.27 \pm 0.18 | 0.36 \pm 0.13 | 0.51 \pm 0.07 | |
| | 0.13 | 4.58 \pm 1.77 | 4.23 \pm 1.24 | 2.92 \pm 0.57 | 27.6 |
| Cr(VI) | 1.3 | 41.47 \pm 20.12 | 36.75 \pm 5.98 | 21.67 \pm 4.97 | 23.1 |
| | 2.6 | 141.89 \pm 50.57 | 97.37 \pm 11.46 | 57.67 \pm 16.34 | 32.0 |
| Cr(III) | 1.3 | 38.89 \pm 26.77 | 26.10 \pm 15.19 | 14.19 \pm 4.57 | 18.7 |
| Cr(VI) + Cr(III) | 1.3 + 1.3 | 159.68 \pm 88.53 | 68.09 \pm 13.99 | 33.18 \pm 8.43 | 28.8 |

Mean amount (\pm SD)

bw: body weight

Table 3. CrI and CrP concentrations over time according to the type of intravenous treatment.

| | Injecte d dose (mg Cr/kg bw) | [CrI] (in mg/L) (Mean ± SD) | | | | | [CrP] (in mg/L) (Mean ± SD) | | | | | |
|---------------------------------|--|-----------------------------|---------------------|---------------------|---------------------|---------------------|-----------------------------|-----------------------------|---------------------|---------------------|---------------------|------------|
| | | At 1h | At 5h | At 24h | At 29h | At 48h | At 1h | At 5h | At 24h | At 29h | At 48h | |
| Contro I | Saline | <LO Q | <LO Q | <LO Q | <LO Q | <LO Q | 0.00 4 ± 0.00 2 | 0.00 2 ± 0.00 2 | <LO Q | <LO Q | <LO Q | |
| | | 0.13 | 0.402 | 0.310 | 0.283 | 0.253 | 0.235 | 0.16 9 | 0.15 1 | 0.072 | 0.063 | 0.037 |
| | | | ± 0.068 | ± 0.037 | ± 0.021 | ± 0.032 | ± 0.010 | ± 0.09 5 | ± 0.05 5 | ± 0.032 | ± 0.028 | ± 0.016 |
| Cr(VI) | 1.3 | 8.391 ± 7.288 | 3.490 ± 0.404 | 2.970 ± 0.413 | 2.799 ± 0.474 | 2.787 ± 0.536 | 6.27 6 ± 4.86 0 | 2.01 0 ± 0.09 3 | 0.891 ± 0.211 | 0.818 ± 0.109 | 0.467 ± 0.110 | |
| | | 2.6 | 6.543 | 3.995 | 4.765 | 4.325 | 4.252 | 3.31 7 | 2.40 4 | 1.434 | 1.235 | 0.748 |
| | | | ± 1.093 | ± 2.305 | ± 1.031 | ± 0.390 | ± 0.465 | ± 0.27 0 | ± 1.37 0 | ± 0.195 | ± 0.234 | ± 0.180 |
| Cr(III) | 1.3 | 0.048 ± 0.010 | 0.023 ± 0.017 | 0.016 ± 0.006 | 0.013 ± 0.005 | 0.011 ± 0.002 | 5.88 1 ± 3.32 4 | 4.37 9 ± 0.96 1 | 1.448 ± 0.885 | 1.744 ± 0.348 | 1.166 ± 0.095 | |
| Cr(VI) + Cr(III) | 1.3 + 1.3 | 3.547 ± 0.267 | 2.930 ± 0.608 | 2.658 ± 0.239 | 2.630 ± 0.527 | 2.502 ± 0.300 | 8.12 0 ± 2.03 7 | 5.68 7 ± 1.53 5 | 3.290 ± 0.609 | 2.515 ± 0.391 | 1.612 ± 0.219 | |

LOQ (CrI) = LOQ (CrP) = 2 µg/L