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Quantitative Analysis of Trace Chromium in Blood Samples. Combination of the Advanced Oxidation Process with Catalytic Adsorptive Stripping Voltammetry

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Abstract

A new method for pretreating blood samples for trace Cr analysis is described. The Advanced Oxidation Process (AOP with H₂O₂ and 5.5-W irradiation for 60 min) is used to remove biological/organic species for subsequent analysis. Prior to the AOP pretreatment, acid (HNO₃) is used at pH 3.0 to inhibit the enzyme catalase in the blood samples. Catalytic Adsorptive Stripping Voltammetry (CAdSV) at a bismuth film electrode (BiFE) gives Cr concentration of 6.0 ± 0.3 ppb in the blood samples. This concentration was confirmed by dry-ashing the blood samples and subsequent analysis by atomic absorption spectroscopy (AAS). This current method may be used to monitor chromium, a trace metal in humans, and the efficacy and safety of chromium supplements as adjuvant therapy for diabetes.

Introduction

Diabetes is a common disease that affects 16 million people in the United States, with an estimated 800,000 new cases diagnosed each year.¹ Chromium is an essential trace element for mammals,² and Cr complexes have been used as a dietary supplement for the treatment of diabetes and its complications.¹ Chromium deficiency has also been linked to increased risk of nonfatal myocardial infarction and cardiovascular disease.³

The frequency of actual chromium deficiency in the population is unknown in part because reliable measures for assessing chromium status in humans are limited.⁴ Since the levels of chromium present in biological tissues and fluids are extremely low (e.g., ca. 3-10 ppb in blood of mammals)⁵ and chromium is bound to peptides,² many difficulties associated with finding a measure of the chromium status are analytical in nature.⁴ Currently there are three analytical techniques with the required sensitivity for the measurement of chromium in biological samples from humans:⁴ neutron activation,³ mass spectrometry (such as ICP-MS),⁴ and graphite furnace atomic absorption spectroscopy.⁶ The first two techniques require expensive instrumentation and are thus not widely available. In the chromium analysis by graphite furnace atomic absorption spectroscopy, the samples are usually heated to, e.g., 2500 °C for pretreatment and atomization.⁶ Heating to that high temperature is needed in part to decompose biological/organic species and to release chromium from their complexes. This technique is highly susceptible to interference from the sample matrix.⁴ In the ICP-MS analysis of chromium in blood and tissue samples, a pretreatment step using microwave digestion is often needed.^{5a,e-g} In addition, quadrupole ICP-MS is not recommended for the ppb-level chromium analysis in biological samples, as a result of interferences from residual carbon.^{5e}

Supporting Information Available

Images of the photochemical reactor and effect of H₂O₂ concentration on CAdSV analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

There is an urgent need to develop improved techniques for the analysis of chromium in biological fluids to assess chromium deficiency and the effect of chromium as adjuvant therapy for Type 2 diabetes.

The Advanced Oxidation Process (AOP) is a combination of hydrogen peroxide and ultraviolet (UV) radiation (or ozone) to generate hydroxyl radicals ($\bullet\text{OH}$),^{7a} and it has been used for the decomposition of organic compounds⁷ and sample pretreatment.^{8,9} Such a process destroys organic matter in environmental or biological samples and releases metals from their complexes prior to chemical analysis.¹⁰

There have been, to our knowledge, few studies of pretreating blood samples by AOP for chemical analysis,^{5a,5-g,11} mostly because blood is a difficult matrix.^{8a} Catalase is an enzyme that decomposes H_2O_2 and protects the body from hydroxyl radicals.¹² The enzyme is, however, detrimental to the AOP treatment of blood samples, and leads to vigorous foaming of the sample. In two papers that have reported pretreating a blood sample through oxidation processes, either a long UV irradiation^{8a} or a different oxidant (potassium peroxydisulfate, $\text{K}_2\text{S}_2\text{O}_8$) was used.^{11b}

We have developed a new process to pretreat blood samples. This new process combines the AOP treatment (aqueous 0.2% H_2O_2 and a 5.5-W UV irradiation) with acid deactivation of the enzyme catalase in blood. This procedure leads to the successful decomposition of biological/organic species in blood, and the conversion of chromium in different oxidation states to chromate. Subsequent Cr analysis by Catalytic Adsorptive Stripping Voltammetry (CArSV), which was recently developed by Wang and coworkers,¹³ yields Cr concentration of 6.0 ± 0.3 ppb in the blood samples. This Cr concentration is consistent with that (6.19 ± 0.03 ppb for the same blood samples) determined by a combination of dry-ashing¹⁴ and atomic absorption spectroscopy. To our knowledge, the current work is a rare, successful AOP treatment of blood samples.¹¹ Our results are reported here.

Experimental

Pretreatment of blood samples by AOP was carried out in a 20-mL photochemical reactor shown in Figure 1. The photoreactor consisted of an outer vessel containing the sample, water-jacketed quartz immersion well, and a UV lamp. (**CAUTION:** UV Light can be dangerous. Wear eye protection). The reactor was designed and built in house, except for the quartz immersion well, which was purchased from Ace Glass. The UV lamp was a 5.5-W quartz low-pressure cold cathode mercury gaseous discharge lamp (Ace Glass 12132-08).

CArSV experiments were conducted using Electrochemical Workstation 650A (CH Instruments, Austin, TX). The cell assembly consisted of a Bi-coated glassy carbon working electrode (3 mm diameter, Cypress Systems, 66-EE047), Ag/AgCl reference electrode (Model CHI111, CH Instruments), and a platinum wire counter electrode. The Bi coating was prepared by a procedure similar to that developed by Wang and coworkers.^{13b} A glassy carbon electrode was polished, exposed to piranha solution (**CAUTION:** Piranha solution reacts violently with organics and proper caution should be taken when handling), and sonicated prior to Bi coating. A 1.245 mg L^{-1} bismuth-0.1 M acetate buffer (pH 4.5) was prepared from a bismuth AA standard solution (1,000 ppm, Aldrich). The Bi coating onto the glassy carbon electrode was conducted at -1.20 V for 120 s. pH measurements were carried out with a pH meter (Accumet Basic, Fisher Scientific). All glassware was soaked in 1 M nitric acid and rinsed several times with deionized water prior to use.

Potassium hydroxide (Certified ACS, Fisher), potassium nitrate (Certified ACS, Fisher), potassium oxalate (Certified ACS, Fisher), acetic acid (glacial, Fisher), nitric acid (70%, Trace Metal Grade, Fisher), sodium acetate (anhydrous, Certified ACS, Fisher), and

diethylenetriaminepentaacetic acid (DTPA, $\geq 99\%$, Fluka) were used as received. H_2O_2 (3%) was freshly prepared from 30% H_2O_2 (Optima Grade, Fisher) and deionized water. **(CAUTION:** acetic acid and nitric acid are corrosive; hydrogen peroxide is a strong oxidizer and is corrosive). Bi and Cr(VI) AA standard solutions (1000 mg/L, Aldrich) were diluted prior to use. The electrolyte solution contained 0.1 M sodium acetate and 0.25 M KNO_3 , and its pH was adjusted to 6.0 with CH_3COOH . The DTPA solution (0.1 M) was prepared by dissolving the DTPA in water while adjusting the pH to 6.0 with 25% NH_4OH (Fisher Scientific). Cr(VI) standards were prepared by diluting the appropriate amount of stock solution in electrolytes. Porcine blood samples were obtained from Wampler's Farm (Lenoir City, Tennessee). The blood was collected in 1-L bottles each containing 2 g of potassium oxalate to prevent coagulation.¹⁶

Pretreatment of blood samples by AOP

A porcine blood sample (0.5 mL) was diluted with deionized H_2O (15 mL) in the photoreactor. Concentrated HNO_3 (2 drops) was added to inhibit the enzyme catalase in blood. The color of solution changed from deep red to deep brown (pH ca. 3.0). After stirring at 23 °C for 5 min, H_2O_2 (3%, 1.0 mL) was added slowly to give the concentration of H_2O_2 in the mixture at ca. 2.0 g/L, and the mixture was then irradiated with the 5.5-W UV lamp for 40 min. The photoreactor was wrapped with aluminum foil during the UV irradiation. KOH was then added to adjust the pH of the solution to 9.5. The UV irradiation was continued for an additional 20 min. The clear, faint yellow solution was analytically transferred to an electrochemical cell. KNO_3 and NaOAc were added such that their concentrations were equal to those of the electrolyte solution. The solution volume was adjusted to 20 mL with deionized water, and the pH was then adjusted to 6.0 by concentrated acetic acid. The sample was then ready for analysis by CAdSV.

When 30% H_2O_2 (1.0 mL) was used in an otherwise identical pretreatment of the blood sample, the process gave a nearly colorless solution. However a subsequent Cr analysis was found to be affected by the excess H_2O_2 , and the best amount of H_2O_2 for the treatment was 3% (1.0 mL) H_2O_2 . These findings are discussed below.

Other alternative AOP procedures were also studied. The procedure below involves a single-step AOP treatment at pH ca. 3.0 for 40 min. The error in the Cr analysis of samples from this shortened step is, however, ca. 10% larger than the procedure discussed above. A porcine blood sample (0.5 mL) was diluted with deionized H_2O (15 mL) in the photoreactor. Concentrated HNO_3 (2 drops) was added to inhibit the enzyme catalase in blood. The color of solution changed from deep red into deep brown (pH ca. 3.0). After stirring at 23 °C for 5 min, H_2O_2 (3%, 1.0 mL) was added slowly, and the mixture was then UV-irradiated for 40 min. The clear, faint yellow mixture was analytically transferred to an electrochemical cell. KNO_3 and NaOAc were added such that their concentrations were equal to the electrolyte solution. The solution volume was adjusted to 20 mL by deionized water, and the pH was then adjusted to 6.0 by concentrated acetic acid. The sample was then ready for analysis by CAdSV.

Adsorptive stripping measurements of Cr(VI)^{13b}

After a Bi film electrode (BiFE) was prepared, standard solutions of Cr(VI) were analyzed by CAdSV. The standards ranged from 52 to 1311 ppt Cr(VI). The solution was first deaerated with nitrogen gas for 5 min. A DTPA solution was then added to a final concentration of 5 mM. Adsorption was carried out by holding the voltage at -0.8 V for 140 s while stirring. Square wave voltammetry (SWV) was performed in an unstirred solution over a potential range of -0.8 to -1.4 V with a potential step of 4 mV, an amplitude of 25 mV, a frequency of 20 Hz, and a quiet time of 8 s. The electrode was cleaned between each measurement by applying -1.2

V potential for 30 sec in the electrolyte solution. The SWVs were baseline corrected by subtraction of the background signal.

Results and Discussion

Pretreatment of Blood by AOP

Chromium is believed to be bound to a peptide whose function is part of an insulin-signaling autoamplification in mammals.^{2b-c} Chemical analysis of chromium in biological fluids is limited by biological/organic species and extremely low concentrations. The most common methods for the pretreatment of blood sample to release the metal ion include dry-ashing or wet-ashing.¹⁴ Dry-ashing involves high temperatures in an oxygen-rich environment, and wet-ashing uses strong acids. Both pretreatment procedures are time consuming and prone to contaminations.

In recent years, the Advanced Oxidation Process has emerged as a powerful method to release metal ions from biological/organic species. The Advanced Oxidation Process combines H_2O_2 and O_3 or ultra-violet radiation to yield hydroxyl radicals ($\bullet\text{OH}$), a very potent oxidant.^{7a} The standard reduction potential of $\bullet\text{OH}$ is 2.8 V. In comparison, the standard reduction potentials of O_3 , H_2O_2 and Cl_2 , three other strong oxidants, are 2.07, 1.78 and 1.35 V, respectively. The products of AOP are water, CO_2 , and free metal ions. Intrigued by the potential use of AOP in blood treatment, we have previously examined and proven the feasibility of releasing of Cr(III) from the biomimetic chromium species chromium(III) propionate.¹⁷

When H_2O_2 was added to a diluted blood sample, vigorous foaming due to the decomposition of H_2O_2 by the enzyme catalase was observed. Although this enzyme protects the body from hydroxyl radicals,¹² the process to decompose H_2O_2 is detrimental to the AOP treatment of blood samples. The effect and the optimal amount of H_2O_2 for AOP treatment are discussed below.

To inhibit catalase activity, several reagents such as copper(II) sulfate, sodium cyanide, sodium azide, nitric acid, sodium-*n*-dodecyl sulphate (SDS), and 3-aminotriazole (ATA) have been used in the literature.¹⁸ In choosing an optimal inhibitor for the deactivation of blood samples in the current work, we considered the following factors: (1) toxicity of the reagents – both NaCN and NaN_3 are extremely toxic; (2) the use of organic inhibitors such as SDS and 3-aminotriazole (ATA) requires their subsequent decomposition in the AOP step; (3) introduction of metal ions (in the use of CuSO_4) to the blood samples that may potentially contaminate the samples; (4) the use of KNO_3 in subsequent electrochemical detection of Cr(VI) by CAdSV.^{13b} We thus chose HNO_3 as the catalase inhibitor in the current work. Our tests revealed that the optimal pH for catalase inhibition was 3.0. To decompose the catalase and prevent the foaming, a 40 min irradiation of the sample under this acidic condition was essential.

Our earlier work on the AOP pretreatment of chromium(III) propionate, a biomimetic chromium species, revealed that the optimal pH for 100% conversion of Cr(III) to Cr(VI) by AOP was 9.5.¹⁷ Therefore, after the initial 40 min irradiation under acidic condition, a second AOP treatment of the blood sample at pH 9.5 for 20 min was used. Direct AOP treatment of blood sample under basic condition, or elimination of the first 40 min irradiation under acidic condition, was found to lead to vigorous foaming. Thus, AOP treatment at low pH followed by subsequent treatment at high pH was found necessary for complete and reproducible Cr(III) to Cr(VI) conversion.

The effect of AOP on the blood sample was visibly evident. When the blood sample was treated with 1.0 mL of 3% H_2O_2 , the bright red mixture turned to a clear, faint yellow solution. The

pretreatment method using 1.0 mL of 30% H_2O_2 in an otherwise identical process turned the blood sample from a bright red mixture to a clear, nearly colorless solution with no observable precipitation (Figure 2). As discussed below, using 1.0 mL of 3% H_2O_2 in the AOP treatment leads to more accurate Cr analyses by the CAdSV process. The procedure with 1.0 mL of 30% H_2O_2 is thus presented as an option.

Measurement of the pretreated blood samples by CAdSV

CAdSV has shown very good sensitivity and low detection limits in the determination of ultratrace concentration of metals.²⁰ The Adsorptive Stripping Voltammetry (AdSV) technique is based on the accumulation of the analyte metal on a suitable working electrode by potential-controlled adsorption and subsequent electrochemical oxidation or reduction of the preconcentrated species. Coupling the adsorptive accumulation with a catalytic action leads to a significant increase of the analytical signal and the sensitivity of detection. Wang and coworkers recently developed the detection of Cr(VI) using Bi film electrode (BiFE) and CAdSV with a detection limit of 15.6 ppt Cr(VI).^{13b} In this procedure, Cr(VI) is reduced to Cr(III) electrochemically on the BiFE, and Cr(III) then binds to DTPA to yield an electroactive species $[\text{Cr}^{\text{III}}(\text{H}_2\text{O})\text{HY}]^-$ ^{20d-e} which is adsorbed onto the BiFE surface. $[\text{Cr}^{\text{III}}(\text{H}_2\text{O})\text{HY}]^-$ is further reduced to $[\text{Cr}^{\text{II}}\text{H}_2\text{Y}]^-$ electrochemically at -1.1 V. Nitrate in the electrolyte solution oxidizes the Cr(II) species back to Cr(III) $[\text{Cr}^{\text{III}}(\text{H}_2\text{O})\text{HY}]^-$ in a catalytic cycle, which significantly increases the SWV signal. Scheme 1 outlines this cycle.^{20d-e}

With the mechanism of the CAdSV(?) technique in mind, it is not difficult to understand the important role of electrolytes in precise CAdSV analyses. As shown in Figure 3, the effect of electrolytes in the CAdSV measurement of the AOP pretreated blood samples was quite clear. CAdSV was directly performed on an AOP pretreated blood sample (**Sample 1**) and a sample *after* the addition of electrolytes (KNO_3 and NaOAc to concentrations of 0.25 M and 0.1 M, respectively) and adjusting the pH to 6.0 (**Sample 2**). Without the electrolytes, the current peak for **Sample 1** is suppressed.

Effects of H_2O_2 on both blood pretreatment by AOP and measurement by CAdSV

One critical factor in AOP is the concentration of H_2O_2 during the UV irradiation. The role of H_2O_2 in AOP includes: (1) decomposition of organic ligands in blood samples, and (2) conversion of Cr at low oxidation states to Cr(VI) species. However, a high concentration of H_2O_2 is not desirable due to its negative influence in blood treatment and measurement. For blood pretreatment using AOP, a large concentration of hydroxyl radicals ($\bullet\text{OH}$) increases the rate of their recombination, thus decreasing the AOP rate.^{7a} Also, for measurements using CAdSV, a large excess of H_2O_2 in solution likely affects the catalytic cycle, as the mechanism in Scheme 1 suggests. The excessive H_2O_2 probably oxidizes the Cr(II) species back to Cr(III) $[\text{Cr}^{\text{III}}(\text{H}_2\text{O})\text{HY}]^-$ and may affect the accuracy of measurement. Thus, several experiments have been conducted to find the appropriate concentration of H_2O_2 which is both sufficient for AOP reaction and acceptable for measurement.¹⁵ Cr(VI) standard solutions (2.6 ppb) containing 0.0 g/L, 1.5 g/L, 3.0 g/L and 8.3 g/L of H_2O_2 were analyzed by CAdSV. The influence of H_2O_2 at 1.5 g/L concentration level was found negligible (Table 1). In separate studies, AOP treatments of blood samples (0.5 mL diluted to 15 mL) were conducted with the H_2O_2 concentrations ranging from 0.75 g/L to 16.65 g/L. Each sample was UV-irradiated in the 20-mL photoreactor for 40 min. After careful screening, 2.0 g/L of H_2O_2 was found to be sufficient for the AOP treatment of the blood samples, and the concentration of H_2O_2 at up to 2.0 g/L does not interfere with the Cr(VI) measurement by CAdSV.

Calibration plot and measurement of blood samples

The SWVs and corresponding calibration plot for Cr(VI) standards measured by CAdSV at a BiFE in the current work are shown in Figure 4. The non-zero intercept on the calibration plot

in Figure 4b is believed to be due to a Faradaic background current as a result of uncatalyzed reduction of nitrate in the absence of the Cr catalyst. With the standard calibration plot in hand, blood samples pretreated using AOP for 60 min (two UV irradiations) and a shorter 40 min (one UV irradiation) were analyzed by CAdSV. The blood samples treated by AOP for 60 min gave Cr concentrations of 6.0 ± 0.3 ppb. The Cr analysis of the sample after the shorter (40 min) AOP pretreatment yielded 5.4 ± 0.3 ppb. As discussed below, dry-ashing followed by AA analysis as well as Cr spiking tests of the same blood sample gave Cr concentration of 6.19 ± 0.03 and 6.1 ± 0.2 ppb, respectively. In other words, the pretreatment using two UV irradiations, first at pH ca. 3.0 for 40 min and then at pH 9.5 for 20 min, gives more accurate Cr(VI) concentration of the blood sample. This finding is in accord with our previous study on the AOP pretreatment of biomimetic chromium(III) propionate.¹⁷

Standard spiking is commonly used in analytical chemistry as a means of method validation. To test the accuracy and sensitivity of our technique, we spiked two blood samples with known amounts of Cr(VI): 260 ppt Cr(VI) and 780 ppt Cr(VI) in separate tests.¹⁵ CAdSV analysis of these two blood samples gave Cr(VI) concentrations of 414 ppt and 927 ppt, respectively (Figure 5). This gives Cr(VI) concentrations in the blood of 6.2 ppb and 5.9 ppb after the subtraction of the spiked standard. These concentrations correspond to the data obtained by direct analysis of the blood, and their average is 97% of the value from dry-ashing and AAS, which is discussed below.

Determination of the chromium concentration in the blood samples by dry-ashing

Dry-ashing and subsequent Cr analysis by AAS of the blood samples was conducted to assess the accuracy of the Cr analysis by combination of AOP with CAdSV developed in the current work.

In the current studies using dry-ashing and AAS, a sample from the blood batch used in AOP (and subsequent analysis by CAdSV) was dry-ashed by heating a 100-mL (100.0303 g) sample slowly and gently on a flame in a large evaporating dish to remove water. After the volume of the sample was decreased by 50%, the temperature of the flame was increased and the sample was heated for 3 h. The sample was then carefully transferred to a quartz crucible and heated for another 2 h until only a small amount of inorganic residue remained. The weight of the final residue with a brick-red color was 0.9386 g. This residue was then dissolved in 10 mL of concentrated HNO_3 . The solution was heated at 70 °C until all solid was dissolved. The solution was heated to concentrate the volume to 1 mL. Atomic absorption spectroscopy was then used for the measurement of the Cr concentration of the solution. The Cr concentration in the blood was found to be 6.19 ± 0.03 ppb.

Conclusion

The current work shows chromium content at the ppb level in blood samples is easily analyzed through the use of the Advanced Oxidation Process (H_2O_2 and 5.5-W UV irradiation), followed by CAdSV using a BiFE. The concentration of 6.0 ± 0.3 ppb chromium in the AOP-treated sample is consistent with that determined by a combination of dry-ashing and AAS. AOP, after the chemical deactivation of the enzyme catalase, offers a fast, clean and inexpensive method for removing organic biological/organic species in blood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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16. Standard blood collection tubes, such as Vacutainer® from Beckton, Dickinson, and Company (www.BD.com), contain 2 mg/mL of potassium oxalate (an anticoagulant) and 2.5 mg/mL of sodium fluoride (a preservative). We modified this standard procedure to contain just the anticoagulant.
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19. Ferric ions $\text{Fe}(\text{H}_2\text{O})_6^{3+}$ in the solution after the AOP treatment may account for the faint color in Figure 2 (right). See the Experimental section for details of this procedure using 30% H_2O_2
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Figure 1. Schematic of photochemical reactor used in AOP experiments.¹⁵

Figure 2.
Blood samples before (left) and after (right) AOP with H₂O₂.¹⁹

Figure 3. Square-wave voltammograms of AOP-treated blood samples with and without electrolytes.

Figure 4.

Square wave voltammograms (top) and corresponding calibration plot (bottom) of Cr(VI) standard solutions measured by CAdSV at a BiFE. The current of the blank was subtracted from those of samples in the calibration plot.

Figure 5. SWVs of AOP pretreated blood samples spiked with 0 ppt (red), 260 ppt (black), and 780 ppt (blue) Cr(VI).¹⁵

Scheme 1.
 Mechanistic pathway in the detection of Cr(VI) species.^{20d-e}

Table 1

The effect of H₂O₂ concentration on CAdSV analysis of standard 2.6 ppb Cr(VI) solutions (total volume: 20 mL)

Solutions	SWV current (μA)
Blank (buffer)	12.75
2.6 ppb Cr(VI)	20.38
2.6 ppb Cr(VI) + 1.5 g/L of H ₂ O ₂	20.54
2.6 ppb Cr(VI) + 3.0 g/L of H ₂ O ₂	31.99
2.6 ppb Cr(VI) + 8.3 g/L of H ₂ O ₂	39.74