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ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF WATER-SOLUBLE CHROMIUM(VI) AND CHROMIUM(III) COMPLEXES IN BIOLOGICAL MATERIALS

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SUMMARY

A high-performance anion-exchange liquid chromatograph coupled to visible-range (370 nm) and UV (280 nm) detectors and an atomic-absorption spectrometer allowed the rapid determination of Cr^{VI} and/or complexes of Cr^{III} in rat plasma, erythrocyte lysate and liver supernatant treated with Cr^{VI} or Cr^{III} in vitro. Cr^{VI} in the eluates was determined using both the visible-range detector and atomic-absorption spectrometer (AAS). The detection limits of Cr^{VI} in standard solutions using these methods were 2 and 5 ng (signal-to-noise ratio = 2), respectively. Separations of the biological components and of Cr^{III} complexes were monitored by UV and AAS analyses, respectively. Time-related decreases of Cr^{VI} accompanied by increases in Cr^{III} complexes were observed, indicating the reduction of Cr^{VI} by some of the biological components. The reduction rates were considerably higher in the liver supernatant and erythrocyte lysate than in the plasma. These results indicate that the anion-exchange high-performance liquid chromatographic system is useful for simultaneous determination of Cr^{VI} and Cr^{III} complexes in biological materials.

INTRODUCTION

Cr^{VI} has been shown to be more toxic than Cr^{III} in animal experiments [1,2] and is known to be particularly dangerous because of its possible carcinogenic effects [3]. The toxicity of Cr^{VI} is considered to be closely related to its reduction to the trivalent state in biological systems. Elucidation of the toxic effects of chromium requires accurate information concerning the fate of the metal, especially Cr^{VI}, in biological systems.

The fate of Cr^{VI} has been studied in plasma [4], blood cells [5,6], liver microsomes [7] and urine [8] in vitro and in bile [9,10] in vivo. The analytical techniques used in these studies have involved gel permeation chromatography, spectrophotometric approaches and polarography. However, some problems

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remain with regard to sensitivity and accuracy when these techniques are applied to determinations of chromium species in in vivo examinations. On the other hand, the recent major advances in high-performance liquid chromatography (HPLC) suggest the possibility of more sensitive and accurate determinations of chromium species in biological systems. Reversed-phase HPLC [11–13] and ion chromatography [14] have been examined for the simultaneous determination of Cr^{VI} and Cr^{III}. In a previous experiment [15], an anion-exchange HPLC system allowed the simultaneous determination of Cr^{VI} and Cr^{III} complexes in a culture medium. In order to apply these techniques to biological systems, further intensive investigations are required.

The purpose of this study was to examine the applicability of the previously developed anion-exchange HPLC method [15] to the simultaneous determination of water-soluble Cr^{VI} and complexes of Cr^{III} in plasma, erythrocyte lysate and liver-soluble components from rats.

EXPERIMENTAL

Apparatus

A fast protein liquid chromatograph equipped with an anion-exchange column (Mono Q HR 5/5) (FPLC system) (Pharmacia, Uppsala, Sweden) was used. The outlet of the column was connected in series to a visible-range spectrophotometer (Uvidec-100-IV with a preparation cell) (Jasco, Tokyo, Japan), a UV spectrophotometer (Pharmacia) and a Model 6000 Zeeman atomic-absorption spectrometer (Hitachi, Tokyo, Japan).

On the basis of the previous findings for Cr^{VI} separation [15], eluting solutions of pH 8.6 with sodium chloride gradients were selected. The starting buffer solution (buffer A) was 20 m*M* Tris–HCl (pH 8.6) (the Tris reagent was specially refined by Nakarai Chemicals, Kyoto, Japan). The eluting buffer solution (buffer B) was buffer A plus 0.5 *M* sodium chloride (standard reagent, Matsunaga Chemical, Fukuyama, Japan). Sodium chloride gradients of the eluting solutions from 100% buffer A to 100% buffer B were produced according to programmes devised previously. A flow-rate of 2 ml/min was used and the sample volume was 150 or 500 μl. The column was operated at ambient temperature in the range 22–27°C.

Reagents

Standard solutions of Cr^{III} were prepared by dissolving $CrCl_3 \cdot 6H_2O$ (analytical-reagent grade, Merck, Darmstadt, F.R.G.) in distilled water immediately before use. A stock solution of Cr^{VI} was prepared at a concentration of 1 mg/ml by dissolving $Na_2CrO_4 \cdot 4H_2O$ (analytical-reagent grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) in distilled water. Standard solutions of Cr^{VI} were prepared by dilution of the stock solution. Chromium concentrations in the standard solutions were determined by acetylene–air flame atomic absorption spectrometry (AAS) at 359.3 nm after addition of dilute nitric acid.

Preparation of biological samples

Blood was taken from the hearts of Nembutal-anaesthetized adult male Sprague–Dawley rats (Clea Japan, Tokyo, Japan) using heparinized syringes. Plasma and erythrocytes were obtained after centrifuging the blood at 2000 *g* for 10 min. Aliquots of the erythrocytes were suspended in 15 volumes or in an equal volume of 20 m*M* Tris–HCl (pH 7.4) buffer and then haemolysed completely using an ultrasonic cell disruptor (Sonifier Model 200, Branson, Danbury, U.S.A.). Erythrocyte lysates (6% and 50%) were prepared by centrifuging the suspensions at 38 000 *g* for 10 min at 4°C.

The liver was removed and given immediate repeated perfusion with ice-cold saline in order to eliminate any contamination with blood. The liver tissues (3 g) were homogenized in 20 m*M* Tris–HCl (pH 7.4) buffer solution containing 0.25 *M* sucrose using a Potter–Elvehjem (Teflon) homogenizer at 800 rpm for 2 min under ice-cold conditions. A 20-ml volume of homogenate was finally obtained. The homogenate was centrifuged at 105 000 *g* for 60 min at 4°C and the supernatant was obtained.

Aliquots (9 ml) of the plasma and erythrocyte lysates were quickly mixed with 1 ml of 50 ppm Cr^{VI} or Cr^{III} solution using a magnetic stirrer after pre-incubation at 37°C for 5 min. An aliquot (9 ml) of the liver supernatant was also mixed with 1 ml of 100 ppm Cr^{VI} or Cr^{III} solution in the same manner. These chromium-treated samples were filtered through 0.2-μm membrane filters (Millex-FG, Millipore, Bedford, MA, U.S.A.) and analysed by HPLC after scheduled intervals of incubation times up to 2 h at 37°C. The volumes of the plasma and erythrocyte lysate injected were 150 μl and those of the liver supernatant were 500 μl. The final pH values of the chromium-treated samples ranged between 7.1 and 7.7.

Analytical methods

Cr^{VI} separated by HPLC was continuously determined by visible-range spectrophotometry at 370 nm and AAS as described previously [15]. The separation patterns of the other chromium species were monitored by AAS. Elution of the biological components in the samples was monitored by UV spectrophotometry at 280 nm.

RESULTS AND DISCUSSION

Separation and determination of Cr^{VI} in standard solutions

The elution patterns of Cr^{VI} in the standard solutions showed sharp peaks in the visible-range and AAS signals, as shown in Fig. 1. The calibration graphs for Cr^{VI} determination by both methods were linear at concentrations less than 5 ppm. The detection limits of Cr^{VI} by visible-range and AAS analyses were 2 ng and 5 ng, respectively (signal-to-noise ratio = 2). The lower sensitivity of the AAS analysis was mainly due to the higher baseline noise.

In this experiment, the determination of free Cr^{III} species was not performed for two reasons. First, Cr^{III} cations easily become associated with a variety of biological components, such as proteins [16–18], nucleic acids [19,20] and low-molecular-weight ligands [21,22]. Second, under the alkaline conditions of the

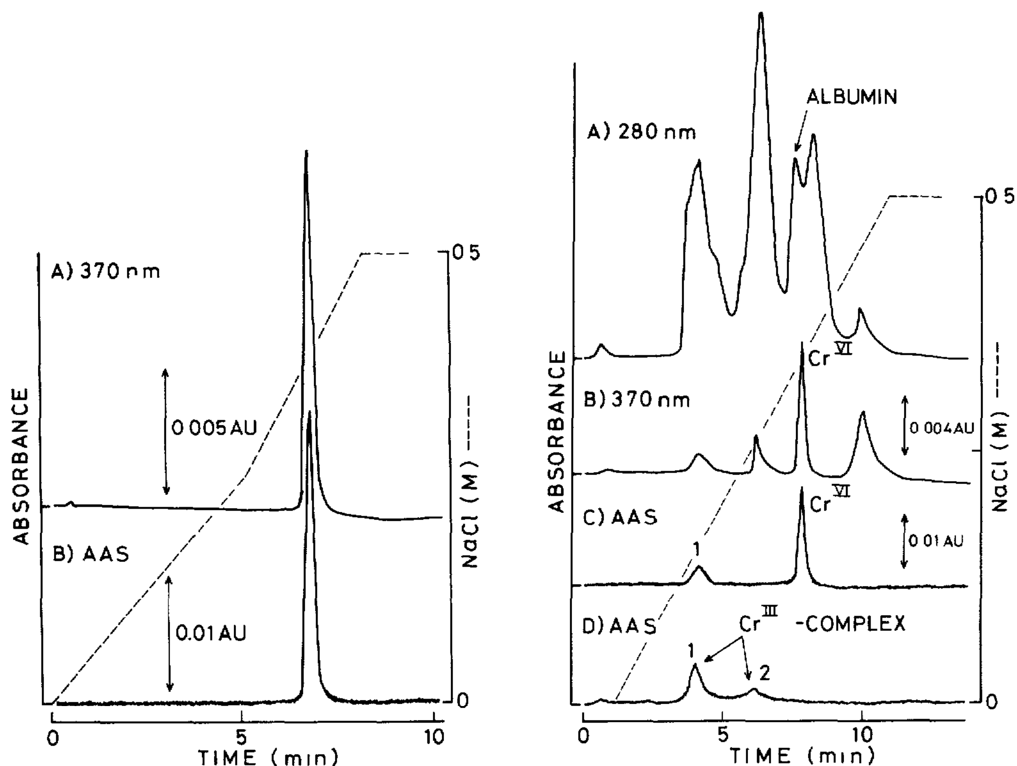


Fig. 1. Elution profiles of 2.5 μg of Cr^{VI} in a standard solution monitored by visible-range (370 nm) and AAS analyses.

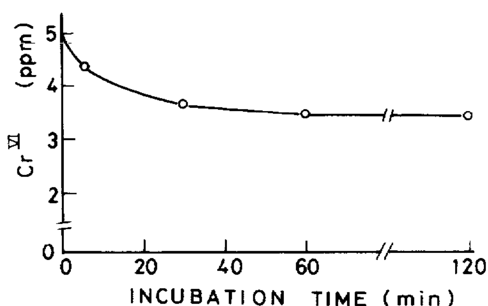
Fig. 2. Anion-exchange HPLC profiles of rat plasma treated with Cr^{VI} (A–C) or Cr^{III} (D). The samples were incubated at 37°C for 1 h after the addition of Cr^{VI} or Cr^{III}. 1, 2 = unidentified Cr^{III} complexes.

eluting solutions used here, water-soluble Cr^{III} is converted into its hydroxides, which exist as colloidal particles and are therefore unsuitable for HPLC. Water-soluble Cr^{III} cations in non-biological samples were quantitatively separated from Cr^{VI} by anion-exchange HPLC after chelation with EDTA [15].

Plasma

HPLC profiles of the plasma samples treated with Cr^{VI} and Cr^{III} are shown in Fig. 2A–D. Elution peaks of Cr^{VI} were clearly shown by both visible-range and AAS analysis (B and C). Slightly before the Cr^{VI} peak, the elution of albumin was monitored by UV analysis (A) [this protein was identified by comparison with the elution pattern of purified bovine serum albumin (Sigma, St. Louis, MO, U.S.A.)]. However, it hardly affected the determination of Cr^{VI} because of the very weak visible absorption of this protein. If complete separation of albumin from Cr^{VI} were to be required, buffer solutions of pH 7.4 would be more suitable. Eluting solutions of lower pH allow the earlier elution of albumin whilst hardly affecting the elution time of Cr^{VI} [15].

In addition to the Cr^{VI} peak, another chromium peak (1) was shown by AAS



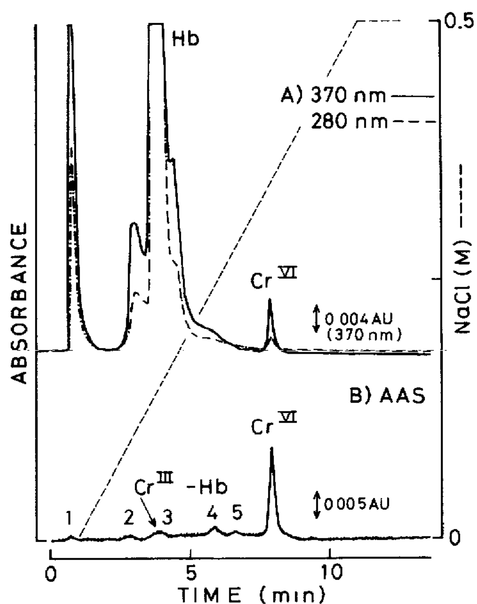


Fig. 3. Reduction curve of Cr^{VI} in rat plasma monitored by anion-exchange HPLC–visible-range analysis. The sample was incubated at 37°C after the addition of Cr^{VI}.

Fig. 4. Anion-exchange HPLC profiles of rat erythrocyte lysate (6%) treated with Cr^{VI}. The sample was incubated at 37°C for 1 h after the addition of Cr^{VI}. 1, 2, 4, 5 = unidentified Cr^{III} complexes.

(Fig. 2C). The recoveries of four measurements of the total chromium in these peaks ranged from 95% to 104% by fractionation AAS analysis [15]. The elution time of peak 1 was coincident with one of the main peaks separated from the plasma sample treated with Cr^{III}, as shown in Fig. 2D. These results suggest that Cr^{VI} was reduced and that the resulting Cr^{III} cations formed complexes with some of the plasma components.

Analytical errors in the Cr^{VI} determination by visible-range and AAS analyses were within ±3% of the mean values. When the Cr^{VI} concentrations in the plasma sample measured by visible-range analysis were plotted against incubation time, a decreasing curve was obtained as shown in Fig. 3, which indicates that the initial Cr^{VI} concentration decreased to a constant level by approximately 1.5 ppm within 2 h. This was caused by reduction, indicating that the reducing capacity of the plasma sample was 1.5 μg/ml Cr^{VI}, consistent with that of human plasma and serum (1.1–1.9 ppm of Cr^{VI}) reported by Korallus et al. [4].

Erythrocyte lysate

Typical HPLC profiles of the erythrocyte lysate (6%) treated with Cr^{VI} are shown in Fig. 4A and B. The elution peaks of Cr^{VI} were detectable by visible-range and AAS analyses. As shown in Fig. 4A, the elution of haemoglobin (Hb) was completely finished before the start of Cr^{VI} elution and no components interfered with the determination of Cr^{VI}. In addition to the Cr^{VI} peak of the AAS

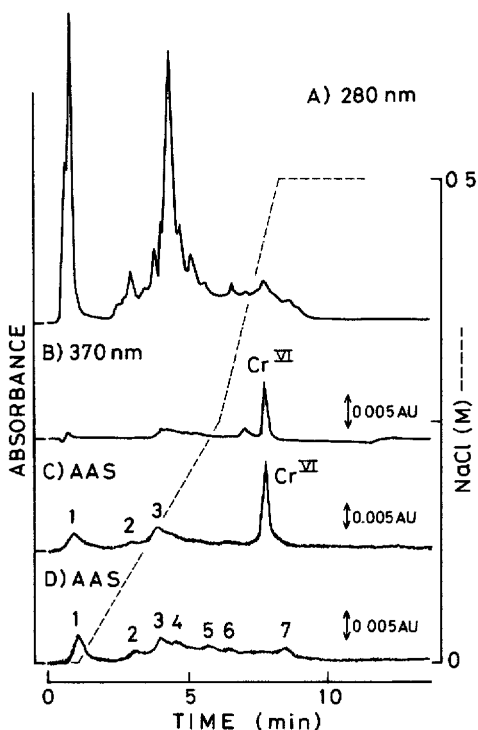


Fig. 5. Anion-exchange HPLC profiles of rat liver supernatant treated with Cr^{VI}. The sample was incubated at 37°C for 5 min (A–C) and 40 min (D) after the addition of Cr^{VI}. 1–7 = Unidentified Cr^{III} complexes.

signal (Fig. 4B), other chromium distributions including five distinguishable peaks were shown by AAS. A comparison between the UV and AAS profiles indicated that the third peak was due to the Cr^{III}–Hb complex. The distribution pattern of chromium (except for Cr^{VI}) was substantially consistent with that of the erythrocyte lysate treated with Cr^{III}, indicating the formation of complexes of some of the erythrocyte components with Cr^{III} reduced from Cr^{VI}. The reduction of Cr^{VI} in the 50% erythrocyte lysate was more rapid than that in the 6% lysate as shown in Fig. 6.

Liver supernatant

Fig. 5 shows the HPLC profiles of the liver supernatant sample treated with Cr^{VI} after incubation for 5 min (A–C) and 40 min (D). Although a number of UV peaks due to soluble components of liver cytosol were seen (A), none of them disturbed the Cr^{VI} determination by visible-range analysis (B). However, the Cr^{VI} level after incubation for 5 min was only 20% of the initial concentration. AAS analysis (C) showed a wide range of chromium distribution, including two clear peaks (1 and 3) and one barely distinguishable small peak (2). After incubation for 20 min, the Cr^{VI} peak almost disappeared and chromium in the other regions increased. The final distribution pattern, including seven chromium peaks

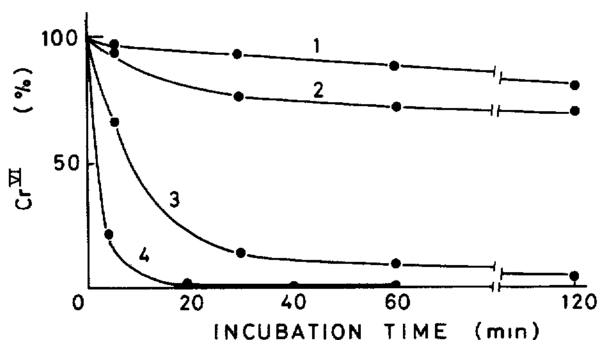


Fig. 6. Relative reduction curves of Cr^{VI} in rat plasma (2), 6% and 50% erythrocyte lysates (1 and 3) and liver supernatant (4). These samples were incubated at 37°C after the addition of Cr^{VI}.

as shown in Fig. 5D, was consistent with that of the Cr^{III}-treated sample, indicating the reduction of Cr^{VI} by some of the liver soluble components.

Relative reduction rates of Cr^{VI} in the three biological specimens studied are shown in Fig. 6. The reduction rates in the liver supernatant and erythrocyte lysate (50%) were very high in comparison with that in the plasma sample.

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