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ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ASCORBIC ACID AND HEXAVALENT CHROMIUM IN RAT LUNG PREPARATIONS AFTER TREATMENT WITH SODIUM CHROMATE IN VITRO AND IN VIVO

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SUMMARY

Simultaneous analysis of ascorbic acid and chromium(VI) in soluble fractions and bronochoalveolar lavage fluids of rat lungs treated with sodium chromate in vitro and in vivo was performed by anion-exchange high-performance liquid chromatography coupled to a photodiode-array detector. Absorbances at 265 and 370 nm were used for the determination of ascorbic acid and chromium(VI), respectively. The calibration graphs of standard solutions were linear in the test ranges of ascorbic acid and chromium(VI) (below 10 and 8 ppm, respectively). The detection limits of ascorbic acid and chromium(VI) were 1 and 0.5 ng, respectively. The recovery of ascorbic acid from lung tissues homogenized at pH 7.4 was 99%, and that of chromium(VI) was 96%, when tissues were homogenized under alkaline conditions (pH 11.4). Using this method, ascorbic acid levels in the soluble fractions and lavage fluids of normal rat lungs were determined. In the lung of a rat intratracheally injected with a saline solution of sodium chromate, ascorbic acid decreased to 80% of the normal level, and ca. 90% of the chromium(VI) was reduced within 4 min after injection, indicating that the ascorbic acid-related reduction of chromium(VI) is very rapid. The present method will be useful for studies of the reduction of chromium(VI) by ascorbic acid in biological systems.

INTRODUCTION

The toxicity of chromium compounds is strongly dependent on the valence states of the metal ion [1]: hexavalent chromium [Cr(VI)] is more toxic than the trivalent species [1–4]. Inhalation of Cr(VI) compounds involves the risk of respiratory cancer [5–7]. The intracellular reduction of Cr(VI), which follows its penetration into cells, is believed to be a prerequisite for chromium toxicity [8]. Recent in vitro experiments using human plasma [9] and bronchoalveolar lavage fluids and lung tissues of rats [10] have demonstrated that ascorbic acid

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(AsA), one of the most abundant biological reductants, plays an important role in the reduction of Cr(VI). In order to investigate further the details of Cr(VI) reduction by AsA in vivo, suitable methods for simultaneous determination of both substances in biological samples are required. Although high-performance liquid chromatography (HPLC) has been widely used for the determination of AsA and its metabolites in biological samples [11–14], it seems to be difficult to use such methods for the determination of Cr(VI) as well as AsA. Recently, Cr(VI) in biological samples has been shown to be easily determined by anion-exchange HPLC [15]. The purpose of the present study was to examine the applicability of HPLC to the determination of AsA and Cr(VI) in the soluble fractions and bronchoalveolar lavage fluids of rat lungs treated with sodium chromate in vitro and in vivo.

EXPERIMENTAL

Apparatus and analysis

The HPLC apparatus equipped with an anion-exchange column (Mono Q, HR 5/5) (Pharmacia, Uppsala, Sweden) used in this experiment was the same as that previously reported [15]. A photodiode-array detector (1040M, Hewlett Packard, F.R.G.) was used. The starting buffer solution (buffer A) was 20 m*M* Tris–HCl (pH 7.4). The eluting buffer solution (buffer B) was buffer A containing 0.5 *M* sodium chloride. Gradients of sodium chloride from 100% buffer A to 100% buffer B were produced by mixing buffers A and B. A flow-rate of 2.0 ml/min was used and the sample volume was 25 μl. The column was operated at ambient temperature in the range 22–27°C. The elution patterns of AsA and Cr(VI) were monitored at 265 and 370 nm, respectively, which corresponded to absorption maxima in the present eluting solution [10,16]. Absorption at 275 nm was also recorded, since this chromium species showed an additional maximum at this wavelength [16].

Reagents

Standard solutions (1–10 ppm) and a stock solution (0.3 m*M*) of AsA were prepared by dissolving L-AsA (reagent grade, Sigma, St. Louis, Mo, U.S.A.) in ice-cold 20 m*M* Tris–HCl buffer (pH 7.4) containing both 0.9% sodium chloride and 0.05% Na_2EDTA as a stabilizer (EDTA saline–Tris buffer). These AsA solutions were kept ice-cold and used as quickly as possible after preparation. Standard solutions (1–8 ppm) and stock solutions (0.6, 3 and 1.5 m*M*) of Cr(VI) were prepared by dissolving sodium chromate tetrahydrate (analytical-reagent grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) in distilled water.

In vitro experiments

Stability test of AsA and Cr(VI) in mixed solutions. Dilute solutions of AsA and Cr(VI) (both 0.03 m*M*) and mixed solutions with different molar ratios of Cr(VI) (0.006, 0.03 and 0.15 m*M*) to AsA (0.03 m*M*) were prepared from the stock solutions, using EDTA saline–Tris buffer, in an ice-cold bath. To investigate the

stability of AsA and Cr(VI), time-related decreases in the concentrations of both substances in these solutions were followed for 3 h.

Preparation of biological samples. Male adult rats of the Sprague–Dawley strain (Clea Japan, Tokyo, Japan), fed on a commercial pelleted diet (CE-2, Clea Japan) and sterilized tap-water ad libitum, were used for the preparation of test samples. After blood had been taken from the hearts of Nembutal-anaesthetized animals and the lungs perfused with ice-cold saline via the pulmonary circulation in situ, the lungs were excised. Tissues (0.3 g) of the lungs were homogenized in 20 ml of 20 m*M* Tris–HCl buffer (pH 7.4) containing 0.9% sodium chloride (saline–Tris buffer) using an ultrasonic cell disruptor (Sonifier Model 200, Branson, Danbury, CT, U.S.A.). The homogenates were centrifuged at 38 000 *g* for 10 min at 4 °C and the soluble fractions were separated. The precipitates were homogenized again and the supernatants were obtained in the same manner. These procedures were carried out under ice-cold conditions as far as possible. The initial and second soluble fractions were analysed for AsA, and the total amounts of lung AsA were calculated.

Excised lungs (without perfusion) were lavaged three times with the saline–Tris buffer as described previously [10]. The recovered fluids were immediately centrifuged at 500 *g* for 5 min at 4 °C. The cell-free bronchoalveolar lavage fluids were analysed for AsA.

Recovery test. Recoveries of AsA from lung tissues by the present analytical methods were ascertained by addition of known amounts of L-AsA to suspensions of the tissues (0.3 g) prior to homogenization. Three homogenate mixtures were prepared: tissue alone, tissue plus 50 μg of L-AsA and tissue plus 25 μg of L-AsA. Recoveries of Cr(VI) were also examined by addition of known amounts (20 μg or 10 μg) of this chromium species to lung tissue suspensions in saline Tris–buffer or in an aqueous solution of 5% sodium carbonate (pH 11.4). All were treated identically and assayed by HPLC according to the procedure described.

In vivo experiments

A diethyl ether-anaesthetized animal of the same strain as described above was intratracheally injected with 0.6 ml of a saline solution of sodium chromate [60 μg of Cr(VI) per animal]. Blood was taken from the heart of the animal, 4 min after injection, with a heparinized syringe, and the lung was removed. The lung tissues were separated into soluble and insoluble fractions by the above-described method, and the former was analysed for AsA and Cr(VI). These operations were performed as quickly as possible. Aliquots of the blood and of the soluble and insoluble fractions were digested in nitric acid with addition of hydrogen peroxide on a hot plate. The residues were dissolved in dilute nitric acid and analysed for total chromium by atomic absorption spectrometry (AAS).

RESULTS AND DISCUSSION

L-AsA and Cr(VI) in standard solutions

Separation and determination. Typical separation patterns of L-AsA and Cr(VI) in the mixed solutions are shown in Fig. 1. The elution peaks of L-AsA and Cr(VI) appear in the regions of a low concentration and the highest concentration of

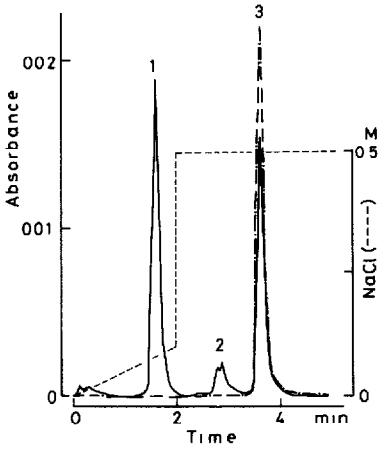

Fig. 1. Anion-exchange HPLC profiles of AsA and Cr(VI) monitored by absorption at 265 nm (—) and 370 nm (—·—), respectively. The sample was a mixture of L-AsA and sodium chromate in 20 m*M* Tris–HCl buffer (pH 7.4), containing 0.9% sodium chloride and 0.05% Na_2EDTA. The sample volume was 25 μl. Peaks: 1 = AsA (0.1 μg); 2 = EDTA and/or its derivatives (unidentified); 3 = Cr(VI) (0.1 μg).

sodium chloride gradient, respectively. The calibration graph for AsA determination by peak area or peak height at 265 nm was linear in the test range of the standard solutions (1–10 ppm). However, the measurements of AsA by peak areas were more accurate than those by peak heights, as indicated by the coefficients of variation (C.V.) of 0.3 and 2.1% ($n=5$), respectively. The calibration graph for Cr(VI) determination by peak area or peak height at 370 nm was linear in the test range of the standard solutions (1–8 ppm). The measurements of both parameters showed almost the same accuracy, as indicated by the C.V. of 0.6 and 0.5% ($n=5$), respectively. The detection limits of AsA and Cr(VI) were 1 and 0.5 ng, respectively.

In the present experiments, the eluates from the column were monitored by absorption at three wavelengths: 265, 275 and 370 nm. Absorption measurements at 265 and 370 nm were most suitable for the detection of AsA and Cr(VI), respectively. In order to analyse low-level Cr(VI) in biological samples, determination at 370 nm is especially desirable, for the reason that this wavelength provides a sharp peak for this chromium anion, with little competitive absorption by biological components. Analysis at 275 nm can be used with a relatively high sensitivity for the determination of both substances in a single chromatogram. Therefore, when sample levels of AsA and Cr(VI) cover a wide range, it is desirable to select a suitable wavelength(s) according to the level.

Stability. As shown in Fig. 2A, L-AsA in the EDTA-containing buffer solutions was sufficiently stabilized for use as a standard for the determination of AsA for at least 3 h after preparation, at ice-cold temperature. In the mixed solutions of AsA and Cr(VI), this vitamin showed a clear decrease with increase of Cr(VI). However, when the molar ratio of AsA to Cr(VI) was more than 1, the decrease in AsA was less than 5% after 1 h. On the other hand, in the samples with high ratios of AsA to Cr(VI), this chromium species was markedly decreased, as seen

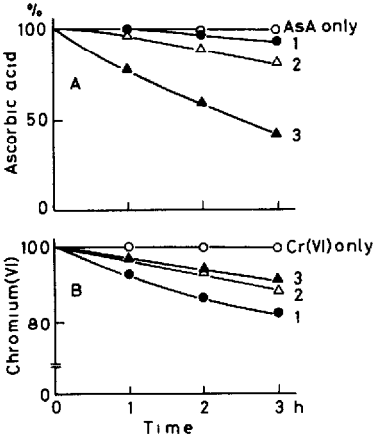

Fig. 2. Decreasing patterns of AsA (A) and Cr(VI) (B) in mixtures with different molar ratios of AsA to Cr(VI) in 20 m*M* Tris–HCl buffer (pH 7.4) containing 0.9% sodium chloride and 0.05% Na_2EDTA, at 0 °C. The molar ratios of AsA and Cr(VI) in samples 1, 2 and 3 were 5:1, 1:1 and 1:5, respectively. The initial concentrations of this vitamin in all the samples and of Cr(VI) in the sample without AsA were 0.03 m*M*. Each point shows the mean of three measurements.

in Fig. 2B. The decreases in these substances were caused by an oxidation–reduction reaction, which was investigated in detail in the previous experiment [10]. Trivalent chromium species reduced from Cr(VI) can be analysed by AAS coupled to the anion-exchange column [16]. In actual inhalation exposure, the ratio of AsA to Cr(VI) in lungs and their lavage fluids would be high. Therefore, the decease of AsA caused by oxidation during the analytical procedure would be negligible, provided that the samples were ice-cold. On the other hand, in biological samples with low Cr(VI) levels, the reducing effect of AsA would result in considerable losses of this chromium species. For protection of Cr(VI) against reduction by AsA, the treatment of samples with sodium carbonate was effective, as shown later.

AsA and Cr(VI) in lung preparations

Separation of AsA and Cr(VI). As shown in Fig. 3A, AsA in the lung soluble fractions was separated as a sharp peak, with an absorption spectrum (Fig. 3B) showing a maximum at 265 nm, consistent with that of standard L-AsA. Although several peaks of biological components appeared, none of these disturbed the AsA peak. As seen in Fig. 3C, in the chromatogram of the soluble fraction of the lung tissues treated with Cr(VI) in vitro, the elution peak of the chromium anions appeared without any interference from biological substances.

In HPLC of the lavage fluids treated with sodium chromate (not shown), AsA and Cr(VI) appeared more exclusively, because of the lower contents of biological components.

Recovery and determination of AsA. AsA in the soluble fractions and lavage fluids (both prepared without EDTA) of the lungs was stabilized naturally to the same extent as L-AsA dissolved in the EDTA-containing buffer, when stored at 0 °C, and high recoveries of AsA were obtained (Table I). AsA levels in the soluble

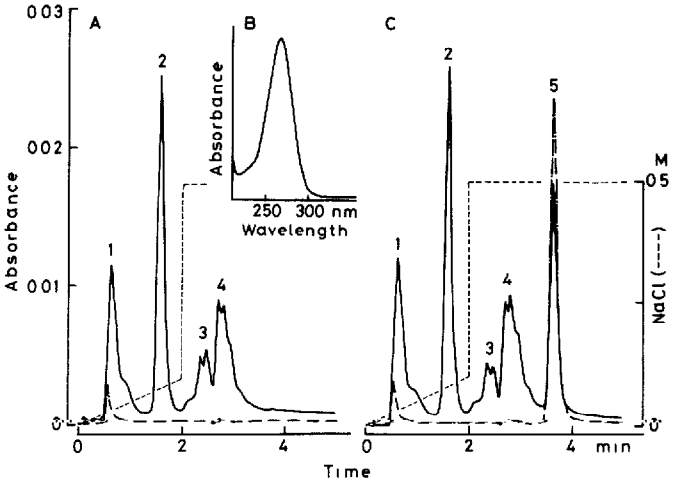

Fig. 3. (A) Anion-exchange HPLC profile of the soluble fraction of rat lung tissues. (B) Absorption spectrum of peak 2. (C) Separation patterns of AsA and Cr(VI) from the soluble fraction of lung tissues treated with sodium chromate in vitro. The eluates were monitored by absorption at 265 nm (—) and 370 nm (—·—). Peaks: 1, 3 and 4 = unidentified components; 2 = AsA; 5 = Cr(VI).

TABLE I

RECOVERY OF ASCORBIC ACID IN RAT LUNG TISSUE

Amount of tissue used (g)	Ascorbic acid (μg)		Recovery (%)
	Added[a]	Measured[b]	
0.3	–	104.4 ± 1.1	
0.3	50	154.1 ± 1.3	99.4
0.3	–	115.2 ± 0.2	
0.3	50	166.4 ± 1.7	102.4
0.3	–	106.2 ± 0.7	
0.3	50	154.8 ± 0.5	97.2
0.3	–	96.5 ± 0.5	
0.3	25	121.1 ± 0.6	98.4
0.3	–	108.4 ± 0.4	
0.3	25	133.6 ± 0.4	100.8
0.3	–	103.8 ± 0.5	
0.3	25	127.9 ± 1.0	96.4

[a]Ascorbic acid was added prior to homogenization of tissue.
[b]Mean ± S.D. of triplicate measurements.

fractions and lavage fluids were 360 ± 30 and 38 ± 5 μg/g of tissue (mean ± S.D. for seven animals), respectively. The former was within the normal range for rat lungs reported by Hornig [17]. The latter was consistent with the previous data [10], which were obtained by the HPLC method reported by Rose and Nahrwold [18].

TABLE II

RECOVERY OF CHROMIUM(VI) IN RAT LUNG TISSUE

Amount of tissue used (g)	Homogenization medium	Chromium(VI) (μg)		Recovery (%)
		Added[a]	Measured[b]	
0.3	20 m*M* Tris-HCl buffer (pH 7.4) containing 9% sodium chloride	20	18.3 ± 0.3	91.1 ± 1.1
0.3	5% Sodium carbonate solution	20	19.3 ± 0.2	96.3 ± 1.1
0.3	5% Sodium carbonate solution	10	9.5 ± 0.1	95.4 ± 1.3

[a]Cr(VI) was added prior to homogenization of tissue.
[b]Mean ± S.D. for five experiments.

Recovery of Cr(VI). Recoveries of Cr(VI) from soluble fractions of the lung tissues treated with sodium chromate in vitro are given in Table II. When the homogenates were prepared with the Tris buffer at pH 7.4, the recoveries were ca. 91%. The low recoveries were caused by the reduction–oxidation reaction between Cr(VI) and AsA during the analytical procedure. In actual samples obtained in inhalation experiments, which are considered to have very low ratios of Cr(VI) to AsA, losses of Cr(VI) induced by the reaction would be considerable. For the protection of Cr(VI) against this reduction, treatment of the samples with sodium carbonate was effective. In the soluble fractions prepared with a 5% sodium carbonate solution (pH 11.4), the ecovery of Cr(VI) increased to ca. 96%. In a chromatogram of the soluble fraction prepared with sodium carbonate (not shown), the original sharp peak of AsA was changed into two broad ones. The denaturation of AsA in the lung tissues caused by treatment with sodium carbonate, probably due to the high pH, might have resulted in higher recoveries. In the AsA-containing lavage fluids adjusted to a pH above 8, no significant loss of Cr(VI) was observed [10]. Thomsen and Stern [19] have also reported that a carbonate-leaching technique is useful for the determination of Cr(VI) in welding fumes.

HPLC profiles of AsA and Cr(VI) after in vivo treatment

Fig. 4 shows HPLC profiles of the soluble fraction of the lung of a rat given an intratracheal injection of a chromate solution [60 μg of Cr(VI) per animal], 4 min after injection. AsA and Cr(VI) were clearly detected by absorption at 265 and 370 nm, respectively. AsA of the soluble fraction showed a decreased level (370 μg per organ), corresponding to ca. 80% of the normal level. Cr(VI) recovered from the soluble fraction was 6.8 μg per organ, only 12.8% of total chromium, which was determined by AAS. The blood concentration of chromium was only 0.26 ppm, indicating that chromium, probably in the hexavalent form, was released into the blood from the lung in only small amounts. In the context of the previous study of the reduction of Cr(VI) in vitro [10], the present observations indicate that the reduction of hexavalent chromium in the lung is very rapid and suggest that AsA present in the lung contributes to this reduction to a great extent. Further detailed in vivo studies of the stoichiometry and chemical kinetics

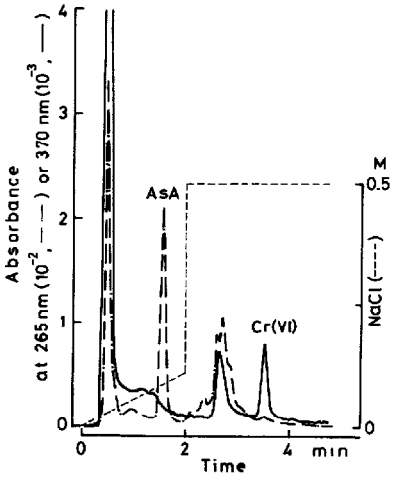

Fig. 4. Anion-exchange HPLC elution patterns of ascorbic acid (AsA) and Cr(VI) in the soluble fraction of the lung of a rat, 4 min after intratracheal injection of sodium chromate.

of the oxidation–reduction reaction between AsA and Cr(VI) in the lung are currently being carried out using the present HPLC method.

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