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Release of Cr(III) from Cr(III) picolinate upon metabolic activation

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Abstract

Hexavalent and trivalent chromium are released into the environment from a number of different industrial activities. It is known that Cr(VI) can be reduced and subsequently complexed by humic acids to produce Cr(III) humic acid complexes in the soil and aquatic environments. The metabolic fate of Cr(III) humic acid complexes and other Cr(III) organic complexes in mammalian systems is unknown. Therefore, Cr(III) picolinate was chosen as a model complex for Cr(III) humic acid complexes and other environmentally relevant Cr(III) complexes. Both human hepatocyte microsomes and primary cultures of chick hepatocytes were used to generate metabolites of Cr(III) picolinate. The results from both of these treatments show that a significant amount of Cr(III) is released (66 and 100%, respectively) and that *N*-1-methylpicotinamide is the primary organic metabolite from this compound.

These data suggest that the populations of humans who are exposed Cr(III) picolinate or other environmentally relevant organic Cr(III) complexes, such as Cr(III) humic acid complexes, are potentially accumulating high levels of Cr(III) intracellularly. This intracellular accumulation of Cr(III) can result in the formation of covalent bonds between Cr(III) and DNA and/or other macromolecules, causing genotoxic effects. These data should be considered when assessing the risk of an area contaminated with chromium.

Keywords: Cr(III) picolinate; Humic acids; Metabolism; Risk assessment

1. Introduction

Chromium is present in significant quantities as an environmental contaminant due to leakage, unsuitable storage, or improper disposal practices in industrial activities. These

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practices have led to many instances where chromium has been released to the subsurface environment. The industrial activities that produce this waste include metallurgy, leather tanning, electroplating, lumber treating, and electricity generation [1].

The most common forms of chromium that are released into the environment include hexavalent and trivalent chromium [1]. Cr(VI) is a strong oxidant that can be reduced to Cr(III) in soils by redox reactions with aqueous ions, electron transfers at mineral surfaces, reaction with simple organic molecules, or reduction by soil humic substances [2]. Humic substances are present in virtually all soils and constitute the major dissolved and particulate organic fraction in most soils [3]. Reduction of Cr(VI) to Cr(III) and subsequent complexation of Cr(III) to humic substances in the soil and aquatic environments has been well documented [4,5].

The complexation of Cr(III) by humic substances plays an important role in this metal's fate, transport and ultimate toxicity in living organisms and should, therefore, be considered an important process in risk assessment. However, the unclear, heterogeneous nature (molecular weight, composition, structure) of metal complexes of humic substances makes prediction of the behavior of the metal difficult. It is known that humic acids are a subclass of humic substances and are comprised predominantly of the functional groups amines, carboxylic acids, carbonyls, phenols, catechols, and quinones; yet the exact structure of humic acids is still unknown [3]. With this in mind, we have chosen the well-characterized Cr(III) picolinate as a model for Cr(III) humic acid complexes found in the environment. Cr(III) picolinate is a compound containing a Cr(III) atom coordinated by three molecules of picolinic acid (Fig. 1). Cr(III) picolinate is an excellent model for Cr(III) humic acid complexes due to similar ligating atoms and complexing geometries in both species. We have used Cr(III) picolinate to model the metabolic fate of Cr(III) humic acid complexes in living organisms.

The toxicity of Cr(III) depends upon its speciation as a salt or organic complex. When Cr(III) is chelated by lipophilic ligands, it is diffusable through cell membranes [6], exerting genotoxic effects both in prokaryotic and eukaryotic cells [6,7]. While these toxicity studies report the intracellular effects of Cr(III) salts and complexes, nothing is known about the metabolic fate of organic Cr(III) complexes in mammalian systems. This is a crucial piece of information that is missing when assembling data for risk assessment of areas contaminated with trivalent chromium. We have determined the metabolites of Cr(III) picolinate produced by human hepatocyte microsomes and primary cultures of chick hepatocytes.

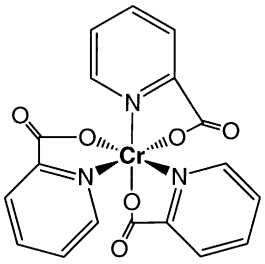


Fig. 1. The structure of Cr(III) picolinate.

2. Methodology

2.1. Materials

All materials used in synthesis ($CrCl_3 \cdot 6H_2O$, picolinic acid, picotinamide, and iodomethane) were purchased from Sigma Chemical Co. and used without further purification. The organic solvents were of high pressure liquid chromatography (HPLC) grade. The human hepatocyte microsomes were purchased from Xenotech lot #062097, pooled from 15 donors (mixed male and female) at 20 mg protein per milliliter. All reagents used in the incubations were purchased from Fisher Scientific and used without further purification.

HPLC data were collected on a Dynamax model SD-200. The absorbance was detected on a model UV-D11 detector. A microsorb-MV^{TM} 100 angstrom C18 column was used for all samples.

2.2. Synthesis of Cr(III) picolinate

Cr(III) picolinate was prepared according to the method described in the literature [8]. Briefly, $CrCl_3 \cdot 6H_2O$ (0.30 mol, 2.8 g) and picolinic acid (0.90 mol, 3.9 g) were added to 35 ml deionized, distilled water and incubated at 37°C for 12 h. The resulting maroon precipitate was filtered, washed, and dried at room temperature. A single peak at 14.8 min using the HPLC method described below was used to verify the purity of this compound.

2.3. Synthesis of N-1-methylpicotinamide iodide

Picotinamide (4.1×10^{-4} mol, 0.050 g) and iodomethane (4.8×10^{-4} mol, 0.030 ml) were added to 15 ml of dry tetrahydrofuran and allowed to reflux for 48 h. A 1 ml aliquot was taken from this solution, diluted with nanopure water to a total volume of 10 ml, and analyzed using HPLC. A single peak at 4.2 min using the HPLC method described below was used to verify the purity of this compound.

2.4. Human microsomal incubation of chromium picolinate

Nine samples were prepared using the following reagents. Cr(III) picolinate (2 μl, 0.25 mM), potassium phosphate buffer (140 μl, 100 mM), nicotinamide adenine dinucleotide phosphate (NADP) (20 μl, 7.65 mg/ml), glucose-6-phosphate (20 μl, 28.2 mg/ml), glucose-6-phosphate dehydrogenase (5 μl, 100 U/ml), and $MgCl_2$ (10 μl, 100 mM) were added to an incubation tube and pre-incubated at 37°C for 3 min. Human microsomal protein (5 μl, 0.1 mg) was added to this solution and the incubation was continued at 37°C in a shaking water bath for intervals of 0 h (three samples, no chromium picolinate added and no incubation time) 1 h (three samples) or 3 h (three samples). The incubations were quenched with 50 μl of acetonitrile. The samples were spun using a Beckman Accuspin FR centrifuge at 10,000 rpm for 10 min and the supernatant removed and stored at −80°C until ready for analysis by HPLC.

2.5. Primary chick hepatocyte cultures

Hepatocyte cultures from 16-day old White Leghorn chick embryos were prepared in 6 cm dishes, at 1.5–2 mg protein per dish and maintained in Williams E medium containing 0.6 μM dexamethasone and 1.8 μM 3,3′,5-triiodothyronine and insulin as described previously [9]. On the second day of culture, the medium was changed to 3.5 ml Williams E medium containing dexamethasone and 3,3′,5-triiodothyronine and Cr(III) picolinate.

Two treatments were conducted with dimethyl sulfoxide (DMSO) alone, two with Cr(III) picolinate at 5 μM, and four with pretreatments of glutethimide (an inducer of cytochrome P450 enzyme 2H1/2) [10] and Cr(III) picolinate. Four treatments were conducted with 3-methylcholanthrene (an inducer of cytochrome P450 enzyme 1A4/5) [10] and Cr(III) picolinate. The concentrations of each of the inducers were 10 or 0.25 mg/ml, respectively. The 6 cm plates were pretreated with the inducers for 16 h before changing medium and adding Cr(III) picolinate at 5 μM for 24 h further incubation. These cells and medium were then frozen for preservation until ready for analysis.

One sample from each treatment was spun using a Beckman Accuspin FR centrifuge at 4°C for 35 min at 10,000 rpm to form a pellet. The media was removed from the tube and 2 ml of water was added to dissolve the remaining pellet. The tube containing this solution was exposed to sonication for 60 s at 25°C. After sonication the sample was spun in the centrifuge at 4°C for 35 min at 10,000 rpm to form a pellet. The supernatant was removed, syringe filtered, and stored for analysis by HPLC.

2.6. Analysis by high pressure liquid chromatography (HPLC)

All samples were analyzed using a Rainin HPLC equipped with a Dynamax model SD-200 pump and an absorbance detector model UV-D11. The absorbance was set at 220 nm for all samples. A Microsorb-MV 100 Å C18 reverse phase column was used for all samples. The mobile phase consisted of 0.1% trifluoroacetic acid/water (solvent A) and 90% acetonitrile/water (solvent B). The solvent gradient was set at 0–30% A → B over 21 min. Hundred microliters of the supernatant from the primary chick hepatocyte cultures, 40 μl of the supernatant from the human microsomal incubations, and 10 μl of the aliquot from the synthesis of 1-methylpicotinamide iodide were used as injection volumes for HPLC analysis.

2.7. Determination of the percent of Cr(III) picolinate metabolized

Standard solutions of Cr(III) picolinate were prepared in order to establish a calibration curve for Cr(III) picolinate using HPLC. Solutions of 5, 50, 100, 150, 200, and 250 μM Cr(III) picolinate were each injected onto the HPLC at volumes of 10, 40, and 100 μl using the method described above. The area under the curve for each peak was determined by software integration. A linear relationship was established for the series of concentrations at a standard injection volume (e.g. at an injection volume of 100 μl, the peak for 50 μM Cr(III) picolinate was integrated to 10 times the value for the peak for 5 μM Cr(III) picolinate). The ratio of the value found from integration of the peak at 14.8 min in the microsomal

assay (at an injection volume of 40 μl) and the value found from integration of the peak at 14.8 min in the standard solution of 250 μM (at an injection volume of 40 μl) allowed for calculation of the percent Cr(III) picolinate that was metabolized.

2.8. Acquisition of mass spectral data

All samples were sent to the Mass Consortium (San Diego, CA) for analysis. The HPLC-MS was performed at the Analytical Core Laboratory, College of Pharmacy, University of Arizona (Tucson, AZ).

3. Results and discussion

3.1. Human microsomal incubation of Cr(III) picolinate

Sixty-six percent of the Cr(III) picolinate from this assay was metabolized in both the 1 and 3 h incubations. There were no differences in the chromatograms obtained from HPLC analysis of the 1 h incubations and the 3 h incubations. We attribute this to the depletion of methylating agents within 1 h of incubation, forming the *N*-1-methylpicotinamide [11]. Each set of chromatograms from these trials showed a predominant peak at 4.2 min for the primary organic metabolite and a peak at 14.8 min for unmetabolized Cr(III) picolinate (Fig. 2).

It is well known that trivalent chromium has a strong tendency to form coordination complexes with a very slow rate of ligand exchange [12]. However, under the metabolic conditions described here, the ligand is readily dissociated from Cr(III). This has significant implications as it suggests that the metabolism of lipophilic forms of Cr(III) (organic complexes) proceed by transport of the Cr(III) complex into the cell, release and transformation of the ligand, and accumulation of Cr(III) in the cells. The Cr(III) is then available to form stable linkages to DNA or other macromolecules found in the cells [12,13], producing genotoxic effects [14]. Such a pathway is outlined in Scheme 1. Our data indicates that this process occurs quickly and results in the release of 66% of Cr(III) from the original complex.

A comparison of the HPLC chromatograms from the 1 h incubation of Cr(III) picolinate with the human microsomes and the control trials (no Cr(III) picolinate incubated with human microsomes) is shown in Fig. 2. This sample was also analyzed using HPLC-MS. A molecular weight of 138 g/mol for the eluent from the predominant peak at 4.2 min was found. The synthesized *N*-1-methylpicotinamide has the same retention time as the predominant peak from these incubations and the same molecular weight as the eluent from this peak (Fig. 3). Therefore, the primary organic metabolite produced by the incubation of Cr(III) picolinate with human microsomal proteins is *N*-1-methylpicotinamide. There is also a shoulder found on this peak that has been identified as nicotinamide. The nicotinamide is a byproduct of the metabolism of the NADP included in the incubation materials. This structure was confirmed by comparison of retention times of commercially available nicotinamide with the sample as well as by analysis by HPLC-MS showing a molecular weight of 123 g/mol for the shoulder.

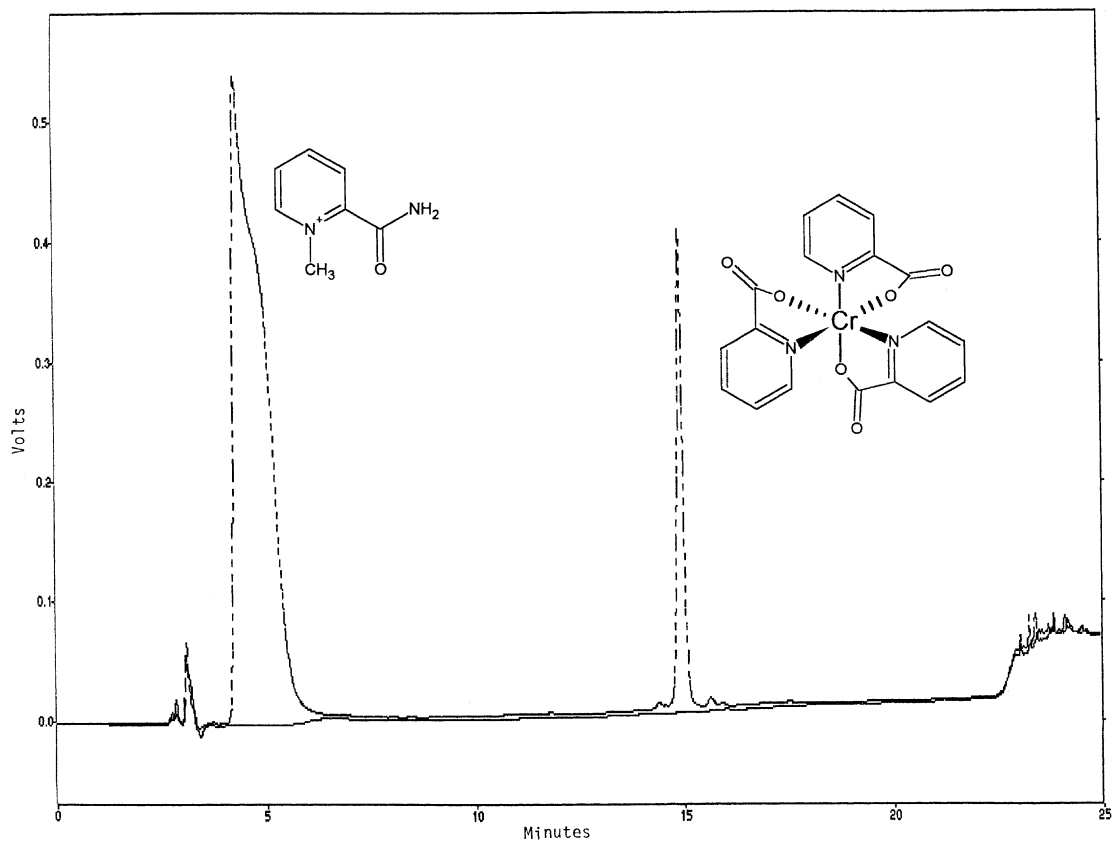
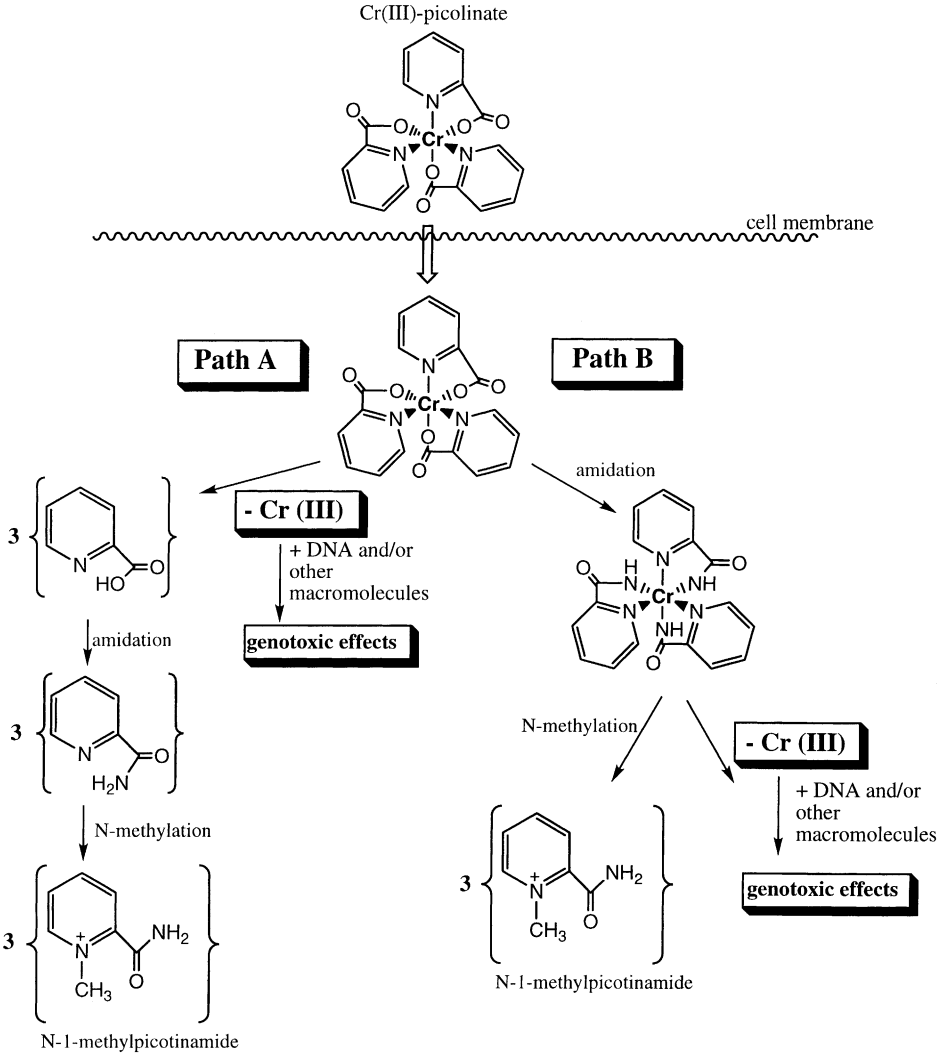


Fig. 2. A comparison of the HPLC chromatograms from the 1 h incubation of Cr(III) picolinate with human microsomes (- - -) and the control incubations (no Cr(III) picolinate) (—).



Scheme 1. Proposed pathways for the metabolism of Cr(III) picolinate. The lipophilic Cr(III) picolinate is able to cross the cell membrane. Path A depicts the immediate release of Cr(III) followed by the biotransformation of picolinic acid to first the amide form and then to the characterized *N*-1-methylpicotinamide as the primary organic metabolite. Path B depicts the amidation of the Cr(III) picolinate, followed by concomitant release of Cr(III) and *N*-methylation of the picotinamide to the primary organic metabolite, *N*-1-methylpicotinamide. In each pathway, Cr(III) is released and available to bind with DNA and/or other cellular macromolecules causing genotoxic effects.

3.2. Primary chick hepatocyte cultures

In order to compare and confirm the results from the incubation of Cr(III) picolinate with human microsomes, primary cultures of chick hepatocytes were employed to generate

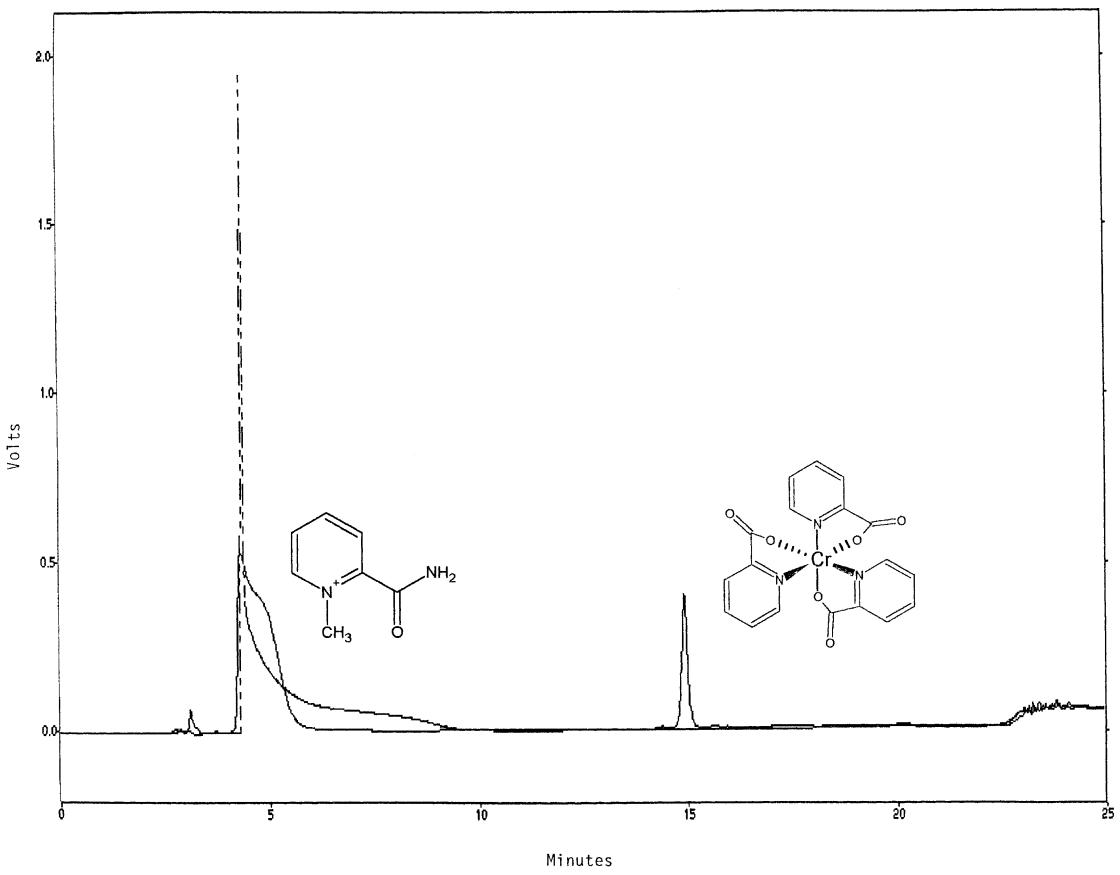


Fig. 3. A comparison of the HPLC chromatograms from the 1 h incubation Cr(III) picolinate with human microsomes (—) and the synthesized *N*-1-methylpicotinamide (- - -).

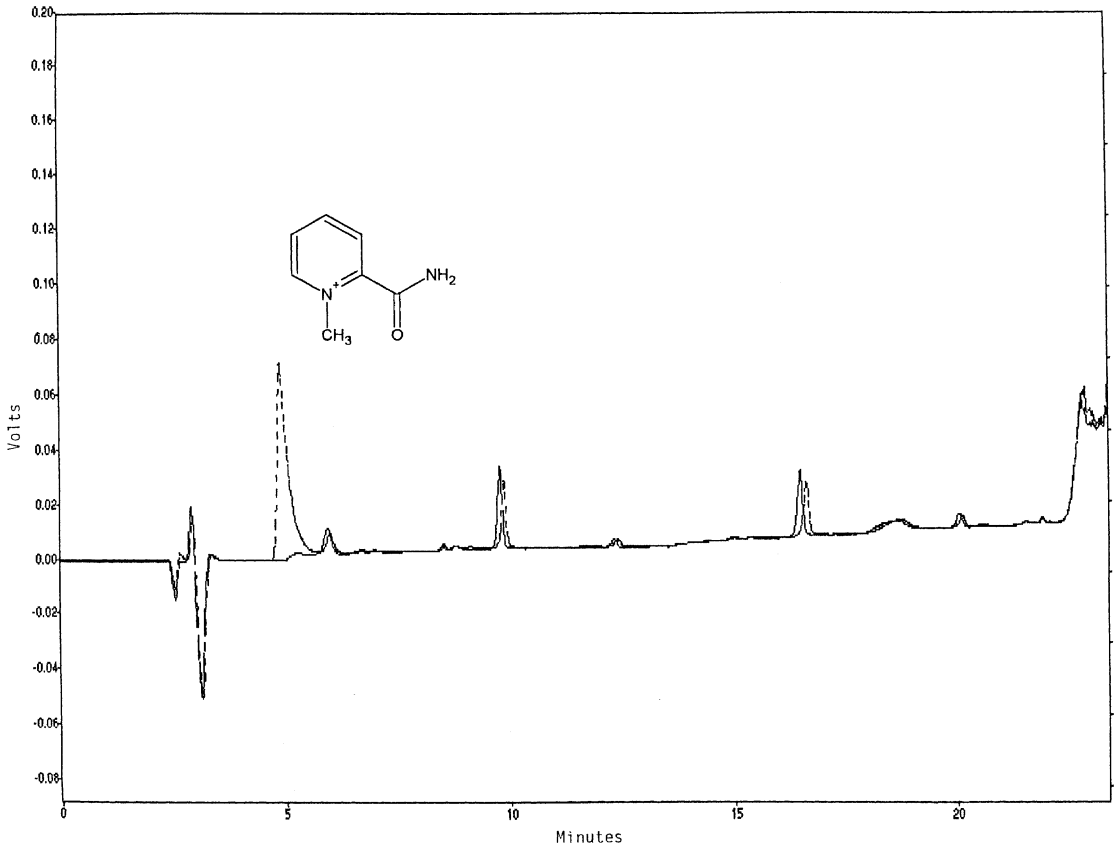


Fig. 4. A comparison of the HPLC chromatograms from the treatment of Cr(III) picolinate with the primary chick hepatocyte method (no inducers) (- - -) and the control treatment (no Cr(III) picolinate) (—).

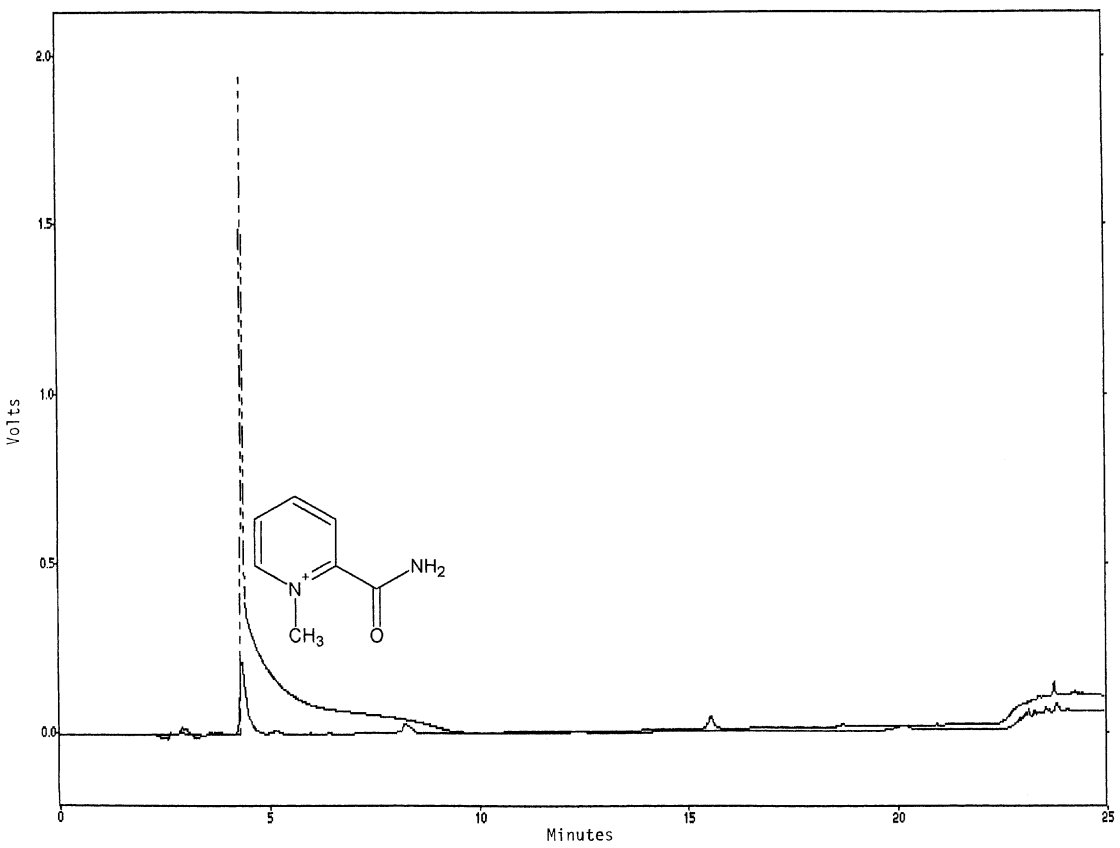


Fig. 5. A comparison of the HPLC chromatograms from the treatment of Cr(III) picolinate with the primary chick hepatocyte method (no inducers) (—) and the synthesized *N*-1-methylpicotinamide (- - -).

metabolites of Cr(III) picolinate. This assay afforded 100% metabolism of the Cr(III) picolinate. All chromatograms obtained from the samples containing Cr(III) picolinate were identical; the inducers did not produce different chromatograms.

The results from the chromatograms collected from the cells treated with Cr(III) picolinate alone and the DMSO control are shown in Fig. 4. The eluent from the peak due to the organic metabolite (at 4.2 min) was collected and analyzed by positive ion mass spectrometry resulting in a positive ion molecular weight of 137 g/mol. This is in agreement with the results from the incubation of Cr(III) picolinate with human microsomes (see above), indicating the production of *N*-1-methylpicotinamide as the organic metabolite of Cr(III) picolinate in these cultures. This was further confirmed by the match in retention times of the synthesized *N*-1-methylpicotinamide and the incubated sample (Fig. 5).

Under these metabolic conditions, 100% of the Cr(III) picolinate is metabolized to *N*-1-methylpicotinamide and Cr(III) is released, presumably by one of the pathways shown in Scheme 1. Once again, the ease of release of Cr(III) in this assay would allow for immediate genotoxic effects.

4. Conclusion

Cr(VI) is released into the environment from a number of different industrial activities. Cr(VI) can be reduced and subsequently complexed by humic acids to produce Cr(III) humic acid complexes in the soil and aquatic environments [4,5]. The metabolic fate of Cr(III) humic acid complexes and other Cr(III) organic complexes in mammalian systems is unknown. Therefore, we have chosen to use Cr(III) picolinate as a model complex for Cr(III) humic acid complexes that would be found in the environment. Both human microsomal and primary cultures of chick hepatocytes were used to generate metabolites of Cr(III) picolinate.

It is well known that trivalent chromium has a strong tendency to form coordination complexes with a very slow rate of ligand exchange [12]. However, under the metabolic conditions described here, the ligand is readily dissociated from Cr(III). This has significant implications as it suggests that the metabolism of lipophilic forms of Cr(III) (organic complexes) proceed by transport of the Cr(III) complex into the cell, release and transformation of the ligand to *N*-1-methylpicotinamide, and accumulation of Cr(III) in the cells. The Cr(III) is then available to form stable linkages to DNA or other macromolecules found in the cells [12,13], producing genotoxic effects [14]. Such a pathway is outlined in Scheme 1.

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Acknowledgements

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