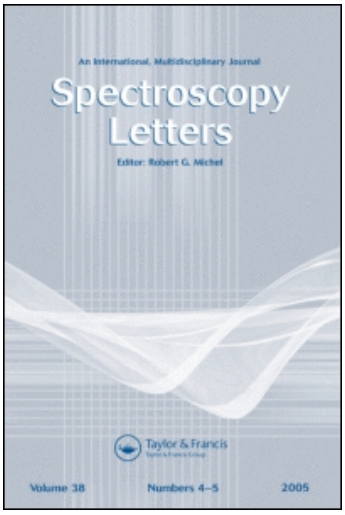

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Use of ^{113}Cd and ^{207}Pb Nuclear Magnetic Resonance Spectroscopy and Flame Atomic Absorption Spectroscopy to Study the Binding of Toxic and Carcinogenic Metals to Calcium Proteins

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ABSTRACT

Flame atomic absorption (FAA) spectroscopy was used to quantitate levels of cadmium (Cd) and lead (Pb) in four mainstream calcium supplements, with ranges of 0.04–0.09 μg/g tablet for Cd and 0.67–0.88 μg/g tablet for Pb being observed. Following this, ^{113}Cd and ^{207}Pb nuclear magnetic resonance (NMR) spectroscopy studies were undertaken to observe competitive binding of both metals in three

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different calcium-binding model systems: calcein (a chelator), casein (Ca^{2+}-rich milk protein), and the calcium-binding protein (CBP) (highly specific for Ca^{2+}). The rationale for this study was to ascertain whether or not the presence of lead and Cd in dietary supplements was to such a degree as to warrant concern by users of the products.

Key Words: Flame atomic absorption; Calcium-binding protein; Nuclear magnetic resonance; Toxic metals; Carcinogen; Estrogen mimic.

INTRODUCTION

The hazards of ingesting lead (Pb) and cadmium (Cd) are well known. Lead has been linked to various mental developmental problems, particularly in young children. With the current maximum threshold of allowable Pb being 10 μg/dL in blood, it has been shown that an increase in blood level Pb concentration from 10 to 20 μg/dL is associated with an average IQ loss of 2–3 points.[1] Cd is a toxic, mutagenic, and carcinogenic metal that has been linked to neurological disorders such as *itai-itai* or "ouch-ouch" disease in the Far East.[2,3] It has recently been implicated in mimicking the effects of estrogen, which can be correlated with a high incidence of hormone-related cancers such as breast cancer and other diseases in many western populations.[4] Both Pb and Cd have also been shown to exhibit immunotoxicity, leading to a diminished immune response.[5] With knowledge of the possible detrimental side effects of consuming these compounds, it is no wonder that concern based on the presence of Pb and Cd in the diet has increased in recent years. One of the major areas of note is that of nutritional supplements, mainly calcium supplements and calcium-containing multivitamins.

In low concentrations, the harmful effects of these compounds are minimal. However, if intake exceeds allowable daily doses, there may be cause for alarm. Expectant and neonatal mothers, as well as individuals of advanced age, are often advised to increase their daily calcium intake through the use of supplements. Unfortunately, one of the major sources of nutritional calcium supplements is ground oyster shells, notoriously high in Pb and other possible trace metal contaminants including Cd.[6] It has been established that both Pb^{2+} and Cd^{2+} can compete with Ca^{2+} in the body when binding to certain calcium-dependent proteins.[5] Therefore, the presence of Pb or Cd in those supplements could lead to the disorders described above as well as others if present in a sufficient concentration.

The following study is a quantitative analysis of Pb^{2+} and Cd^{2+} levels in a series of calcium supplements using flame atomic absorption (FAA) spectroscopy, followed by observations of these metals competitively binding at sites where Ca^{2+} is the native metal cofactor using ^{113}Cd and ^{207}Pb nuclear

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magnetic resonance (NMR) spectroscopy. Results of the FAA analysis will show that, in general, the levels of Pb and Cd in nutritional calcium supplements seem to be acceptable. However, findings of the NMR studies will show that, if Pb or Cd contaminants reach an appreciable level, competition between these metals and calcium for calcium-binding sites on proteins can occur and should be a point of concern.

EXPERIMENTAL

Reagents

Calcein, casein, and calcium-binding protein were purchased from Sigma. Cadmium chloride ($CdCl_2$), lead acetate ($Pb(C_2H_3O_2)_2$), Trizma®, and deuterium oxide were purchased from Aldrich. Over the counter calcium supplements were purchased at a local pharmacy. All chemicals were used without further purification.

FAA Spectroscopy

Sample Preparation

Samples were prepared for FAA analysis by dissolving one tablet of four representative OTC calcium supplements in 20 mL dilute HNO_3 followed by incubation at 37°C to ensure maximum dissolution. Samples were then diluted with H_2O to 1 L and filtered for purity prior to analysis.

Experimental

FAA data were collected with a Varian SpectrAA 220 Fast Sequential spectrometer using an air/acetylene mixture at a flow rate of 13.5/2.00 L/min. Absorbance values were collected in triplicate for both Pb and Cd at 217.0 and 228.8 nm, respectively. Cd and Pb concentrations were quantified by comparison with standard solutions prepared in 10% $HClO_4$.

NMR Spectroscopy

Sample Preparation

Samples of 1 M $Pb(C_2H_3O_2)_2$ and 1 M $CdCl_2$ were prepared by dissolving the appropriate amount of solute in 50 mM buffer (TRIZMA®), pH 7.0. NMR samples were prepared by combining 500 μL aliquots of the appropriate

sample with 50 μL of D_2O. Protein/chelating agents (1 mg/ml solutions) were added to each sample in 10 μL aliquots until a final volume of 650 μL or complete loss of signal was achieved.

Experimental

Spectra were collected using a Bruker Avance 200 MHz NMR with a 5 mm BBO probe tuned to ^{207}Pb or ^{113}Cd and using a composite pulse proton decoupled (CPD) experiment selective for each nuclei. Spectra were collected using 16 scans and the appropriate sweep width for the nuclei under observation ($\pm$ 3000 ppm for Pb^{2+} and $\pm$ 600 ppm for Cd^{2+}). Samples were run locked on D_2O with no internal reference. All spectra were collected at 293 K. Representative spectra for both 1 M $Pb(C_2H_3O_2)_2$ and 1 M $CdCl_2$ are given in Fig. 1.

RESULTS AND DISCUSSION

FAA Spectroscopy

The first phase of this project involved analyzing a series of over-the-counter vitamin supplements for the presence of Cd^{2+} and Pb^{2+} by using FAA spectroscopy. Four types of supplements were chosen for this study: a general multivitamin, a calcium supplement derived from oyster shell, a neonatal vitamin, and a general calcium supplement. Separate analyses were performed on each tablet for the presence of (i) Cd^{2+} and (ii) Pb^{2+}. The results of these analyses indicate that the largest amount of Cd^{2+} ($\sim$0.092 μg Cd^{2+}/g tablet) and Pb^{2+} ($\sim$0.884 μg Pb^{2+}/g tablet) were observed in the general multivitamin and the oyster shell calcium, respectively. The smallest amount of both Cd^{2+} and Pb^{2+} ($\sim$0.041 μg Cd^{2+}/g tablet; $\sim$0.672 μg Pb^{2+}/g tablet) occurred in the same supplement, a general calcium tablet. Results for all four supplements are shown in Table 1. FDA standards indicate that the maximum tolerable daily intake of Cd should not exceed 55 μg, and the daily intake of Pb should not exceed 75 μg.[7,8] Based on these levels, it appears that calcium supplements do not represent a significant source of these contaminant metal ions in the diet.

NMR Spectroscopy

The second phase of this project involved observing the binding of both Cd^{2+} and Pb^{2+} to a series of molecules selective for Ca^{2+} ions. Chosen for analysis were three different classes of calcium-specific molecules. The

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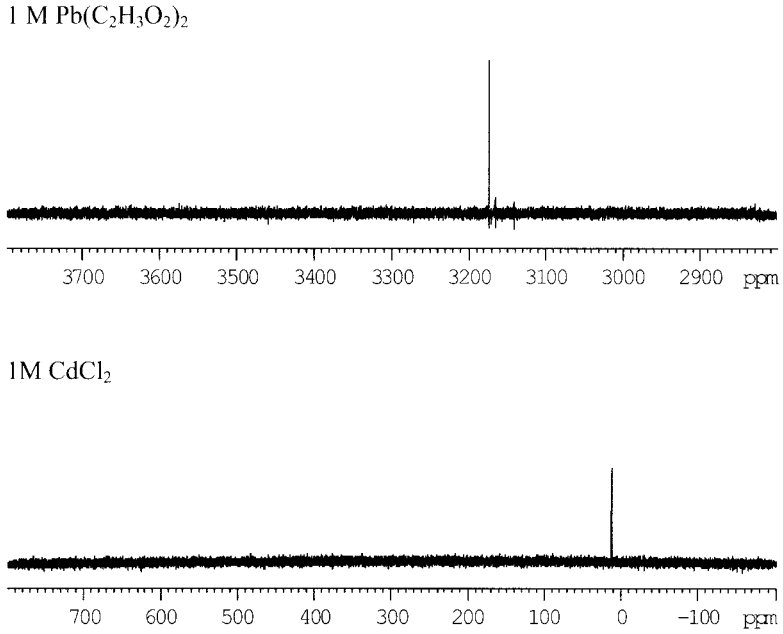

Figure 1. Representative NMR spectra for 1 M $Pb(C_2H_3O_2)_2$ and 1 M $CdCl_2$.

first, calcein, is a small metal chelator that is routinely used as a fluorometric detector for the presence of calcium. It is relatively nonspecific in that it has been shown to bind to other 2 + ions such as Fe(II) as well.[9] The second was technical grade casein, a mixture of several Ca^{2+} specific proteins (α, α_2, β, κ) derived from bovine milk and ranging from 19 to 25 kDa in size.[10] Casein represents approximately 80% of the proteins in milk and binds Ca^{2+} to an extent that is roughly proportional to the number of phosphodiester bonds

Table 1. Levels of cadmium and lead four types of calcium supplement tablets.

Types of supplement	Concentration of cadmium (μg Cd/g tablet)	Concentration of lead (μg Pb/g tablet)
Multivitamin	0.092	0.825
Oyster shell calcium	0.054	0.884
Prenatal	0.043	0.822
General calcium	0.041	0.672

Table 2. Titration of 1 M $Pb(C_2H_3O_2)_2$ with calcein.

Chemical shift (ppm)	Calcein added (μL)	Peak intensity (NMR units)	Peak intensity (%)
3173.30	0	15,1914	100
3173.27	50	42,449	27.9
3173.24	100	26,006	17.1

Note: ppm: parts-per-million.

that are present. The third and most specific calcium-binding molecule chosen was the CBP (roughly 25 kDa in size), derived from vitamin D-induced bovine mucosa and highly selective for Ca^{2+} ions.[11]

Results of all three models show that each type of calcium-specific molecule displays an affinity for both the Cd^{2+} and Pb^{2+} ions in solution. As shown in Table 2, titration of 1 M $Pb(C_2H_3O_2)_2$ with 50 μL aliquots of calcein results in a dramatic decrease in the intensity of the Pb resonance, indicating an increased correlation time due to a shift from free ionic state to bound state. Upon adding the first aliquot, the intensity of the Pb resonance not only decreases by ~72%, but also the appearance of multiple minor Pb resonances is seen, suggesting that Pb may be present as a mixture of different species in solution (not shown). Finally, after the addition of 100 μL of calcein, the Pb resonance is almost completely gone (down to ~17%) indicating that it is bound to the chelator (no precipitate was apparent, indicating that Pb was still in solution) leaving minimal free Pb ion in solution. Similar results are shown for Cd, with the first 50 μL aliquot of calcein causing a decrease in the intensity of the Cd resonance in 1 M $CdCl_2$ by ~78%, and the second 50 μL aliquot eliminating the resonance completely (Table 3). Visually, binding of both Pb and Cd to the calcein fluorophore was apparent based on color change (fluorescent green).

Table 3. Titration of 1 M $CdCl_2$ with calcein.

Chemical shift (ppm)	Calcein added (μL)	Peak intensity (NMR units)	Peak intensity (%)
15.92	0	23,3338	100
14.45	50	51,951	22.3
Not observed	100	0	0

Note: ppm: parts-per-million.

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Table 4. Titration of 1 M $Pb(C_2H_3O_2)_2$ with casein.

Chemical shift (ppm)	Casein added (mL)	Peak intensity (NMR Units)	Peak intensity (%)
3,172.9	0	162,152	100
3,172.8	50	51,718	31.9
3,172.8	100	15,020	9.3

Note: ppm: part-per-million.

Table 4 illustrates the titration of 1 M $Pb(C_2H_3O_2)_2$ with casein, a mixture of Ca^{2+} specific proteins found in milk. As seen, the first 50 μL aliquot results in a decrease of the intensity of the Pb resonance as before. A total of 100 μL of casein almost completely eliminates the resonance. In the case of $CdCl_2$ titrated with casein, the results are much more dramatic. As shown in Table 5, the first 50 μL aliquot of casein almost completely eliminates the Cd resonance, whereas the second 50 μL aliquot does so. This suggests that casein selectively binds to Cd more extensively than to Pb, implying that Cd contamination could pose a serious problem if found in milk.

The final model involved a study of the bovine calcium-binding protein, highly selective for Ca^{2+}. Table 6 illustrates the titration of 1 M $Pb(C_2H_3O_2)_2$ with successive 10 μL aliquots of calcium-binding protein. As can be seen, the intensity of the Pb resonance diminishes steadily upon addition of calcium-binding protein. Finally, after a total of 90 μL of calcium-binding protein has been added, the Pb resonance disappears completely. Table 7 shows a similar trend for the titration of 1 M $CdCl_2$ with calcium-binding protein. Similarly to Pb, the Cd resonance decreases dramatically by the addition of successive aliquots of calcium-binding protein, with the intensity of the resonance at the tenth 10 μL aliquot being only ~9% of the starting intensity; however, unlike the case of Pb, the resonance never truly disappears, suggesting

Table 5. Titration of 1 M $CdCl_2$ with casein.

Chemcial shift (ppm)	Casein added (mL)	Peak intensity (NMR Units)	Peak intensity (%)
15.914	0	22,4279	100
14.792	50	11,761	5.2
Not observed	100	0	0

Note: ppm: part-per-million.

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Table 6. Titration of 1 M $Pb(C_2H_3O_2)_2$ with calcium-binding protein.

Chemical shift (ppm)	CBP added (μL)	Peak intensity (NMR units)	Peak intensity (%)
3,172.4	0	57,567	100
3,172.3	10	54,807	95.2
3,172.3	20	36,558	63.5
3,172.3	30	36,554	63.4
3,172.3	40	36,557	63.5
3,172.3	50	34,293	59.6
3,172.2	60	33,414	58.0
3,172.2	70	29,751	51.7
3,172.2	80	23,099	40.1
Not observed	90	0	0

Note: ppm: part-per-million.

that even though binding between the calcium-binding protein and Cd occurs, the duration of the binding may be shorter than that with Pb.

CONCLUSION

Results of this study have shown that both Pb^{2+} and Cd^{2+} are quite capable of binding to proteins that are selective for Ca^{2+}. Those proteins

Table 7. Titration of 1 M $CdCl_2$ with calcium-binding protein.

Chemical shift (ppm)	CBP added (μL)	Peak intensity (NMR Units)	Peak intensity (%)
11.546	0	149,748	100
11.699	10	143,743	95.9
11.527	20	104,342	69.7
11.424	30	88,896	59.4
11.271	40	70,911	47.4
11.149	50	71,955	48.1
11.057	60	68,248	45.6
10.965	70	65,171	43.5
10.874	80	61,824	41.3
10.782	90	56,681	37.9
10.660	100	13,169	8.8

Note: ppm: parts-per-million.

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that are more selective for calcium, such as casein and the calcium-binding protein, seem to have a tendency to bind to these ions to a greater extent than those that are less selective. In addition, it appears as if Cd binds more readily than Pb, making monitoring of the level of that particular ion in supplements slightly more important than that of Pb, particularly in light of its neurotoxic and carcinogenic properties. However, since both ions can compete with calcium and have been shown to have detrimental effects, both should be strictly monitored. In terms of the level of both metals in current over-the-counter dietary supplements, studies from a limited array of supplements have shown that levels are well below the recommended FDA levels and currently seem to not pose a major health risk.

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