BioMetals 1995, **8**, 105–110

X-ray fluorescence in the assessment of inter-elemental interactions in rat liver following lead treatment

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Received 10 November 1993; accepted for publication 27 May 1994

Energy dispersive X-ray fluorescence technique was employed to study the interactions of lead (50 and 100 mg/kg body wt) with K, Fe, Cu, Zn, Br and Rb in rat liver. Lead was administered orally to rats daily for dosage periods of 1 and 4 months (short and long terms). Hepatic Fe levels were found to increase significantly with the supplementation of low and high doses of lead for both the treatment periods, although the increase was more pronounced following long-term treatment. The levels of hepatic K, Cu and Br were seen to decrease significantly over both time intervals. Moreover, hepatic Rb contents were lowered with the short-term supplementation of low doses of lead. In contrast, Rb and Zn levels were increased when lead was administered for the longer period at both dose levels.

Keywords: energy dispersive X-ray fluorescence, liver, lead toxicity, inter-elemental effects

Introduction

Trace elemental analysis of biological tissues is of increasing interest in connection with the investigation of environmental pollution. Such studies often require multi-element determination with extremely low limits of detection. Techniques for such elemental analysis include atomic absorption, flame photometry, neutron activation and X-ray fluorescence and emission spectrophotometry (Scott 1970, Combs 1974).

Exposure to heavy metals results in a variety of biochemical alterations in liver. Many of these effects result from the capacity of metals to bind to nucleophilic sites in the cell, which primarily occur in the form of free sulfhydryl groups (Eaton *et al.* 1980).

Lead is a ubiquitous environmental toxin and its toxic effects on biological systems have been extensively reported (Baloh 1974, Klein 1974, Stofen 1974). Hepatic lead toxicity results in the alterations of various biochemical parameters in relation to hepatic functions (Scoppa *et al.* 1973, Singh *et al.* 1992, 1994).

Various reports describe the interactions of lead with other elements (Finelli *et al.* 1975, Ragan 1977, Kumar *et al.* 1991). Lead ingestion can reduce the nutritional efficacy of zinc and copper by causing a decrease in the absorption of these nutrients (El-Gazzar *et al.* 1978, Pettering 1978, Finelli & El-Gazzar 1977). It can also lead to a reduction in the biochemical and cellular functions of zinc and copper and thus alter metabolic actions related to hemopoiesis, cellular respiration and other vital functions (Mahaffey & Radder 1980). The interactions between lead, zinc and copper may result in the retardation of body growth, may produce cardiomyopathy and hypertension and might even interfere with iron metabolism (Klauder & Pettering 1975, Flanagan *et al.* 1979). Furthermore, exposure to lead in the form of vehicular exhaust has been shown to interact with Fe, Cu, Zn, K, Rb and Br species in biological samples (Kumar *et al.* 1991, Singh *et al.* 1994). There is, however, a clear lack of information about the dose and time dependent interactions of lead with different elements in rat liver.

Interactions among the trace elements are prevalent and so influential biologically, that nutritional and toxicological studies carried out with single elements might project an inconclusive picture if the levels of interacting elements in dietary samples and body tissues were not also known. In addition, a non-destructive method of analysis would be advantageous since the samples could then be preserved for further investigations. Energy dispersive X-ray fluorescence (EDXRF), a multi-elemental analysis technique, meets all these requirements adequately and has great potential in biology and medicine. This technique provides a fast, reproducible and accurate means of analysis with minimum labor of specimen preparation.

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With advances in experimental facilities, the values of the different parameters involved in EDXRF, such as photoelectric cross-sections, mass absorption coefficients and incident radiation intensity, can be computed with better accuracy.

The aim of the present study was to use EDXRF to explore the possible biological significance of chemical similarities among various elements in rat liver following lead supplementation.

Materials and methods

Animal grouping and dosing

Female Porton rats weighing 140–150 g were obtained from the central animal house of Panjab University and acclimatized in the department animal house for 1 week prior to lead treatment. The animals were randomly distributed into two groups: control (group I) and lead-treated (group II). The lead-treated group was further divided into four sub-groups.

For the two low-dose subgroups, lead acetate was administered orally every day using intubation gavage technique at a dose level of 50 mg/kg body weight for either 1 month (short-term) or 4 months (long-term). For the high-dose sub-groups lead acetate was administered at 100 mg/kg body weight for the same time periods. All the animals were fed pelleted diet (Hindustan Lever Ltd, India) and water *ad libitum* during the study.

The animals were anesthetized under light ether anesthesia, the peritoneal cavities were cut open and the livers were removed. The livers were then perfused with normal saline to remove the blood traces completely. These samples were oven-dried at 70 °C to a constant weight and ground in an agate pestle and mortar. The powdered moisture-free samples were weighed and mixed thoroughly with equal amount of cellulose (Sigma) as a binding agent. The sample–cellulose mixture was then molded into self-supporting pellets of 2.5 cm diameter using an indigenous pure steel die. A constant and uniform pressure of 1055 kg cm^{-2} was applied to the die head by using hydraulic press (Perkin Elmer) so as to get pellets of uniform thickness (75 mg cm^{-2}) below the critical thickness (Kumar *et al.* 1989).

Elemental analysis using EDXRF technique

Elemental analysis of the liver samples was carried out with the help of an EDXRF spectrometer. The experimental arrangement used in the present study is shown in Figure 1. In this arrangement an annular source of ^{109}Cd (20 mCi) and an exciter system (New England Nuclear, Boston, MA, USA) have been used for direct excitation of characteristic X-rays of the elements present in the samples. The average energy of the exciting Ag K X-rays was calculated to be 22.6 keV by considering the weighted average of the Ag K_α and K_β lines according to their intensity ratios. The details of the EDXRF set-up have been described previously (Garg *et al.* 1984).

The samples were run for their characteristic X-ray spectra and the spectra were recorded with a Si(Li) detector (FWHM = 180 eV at 5.9 keV) coupled to a PC-286-based multichannel analyzer (Model S100, Canberra) through a spectroscopy amplifier (ORTEC 572). Each sample was irradiated for time intervals ranging from 20 000 to 50 000 s. The X-ray spectra so obtained were analyzed to find the area of different X-ray peaks using the computer program AXIL (Van Espen *et al.* 1989).

Quantitative estimation

In general, the method used for the quantitative X-ray spectrometry at the early stage was an internal standard method, in which many calibration standards are normally required to adequately cover the wide range of elements present in a given sample with varying concentrations. In practice, the requirement of calibration standards is one of the greatest problems which the analyst is likely to encounter. An alternative method which overcomes this difficulty is the 'fundamental parameter approach' (Gedcke *et al.* 1982). This method uses certain equations relating the measured X-ray counting rate for the given element to the elemental concentration. In case of radioisotope-excited X-ray fluorescence technique such an equation can be written:

$$m_j = \frac{n_{ij}}{I_0 G \varepsilon_{ij} \sigma_{ij} \beta_{ij}} \qquad (1)$$

where m_j is the concentration of the element in g cm^{-2}

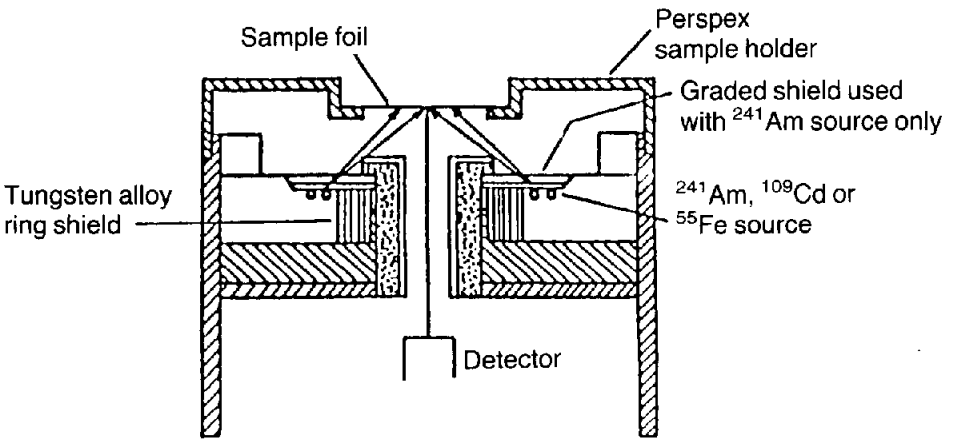

Figure 1. Sample, exciter and detector geometrical arrangement.

present in the sample, n_{ij} is the net counts per unit time for the ith group of X-rays of jth element, I_0 is the intensity of incident photons, G is the geometry factor, ε_{ij} is the efficiency of the detector for the ith group of X-rays of jth element, σ_{ij} is the X-ray fluorescence cross-section of the ith group of X-rays for the jth element and β_{ij} is the self-absorption correction factor of the target material which accounts for the absorption by the target of the incident photons and the emitted characteristic X-rays lying under the ith peak of jth element.

The factor I_0G in equation (1) represents the intensity of exciting radiations incident on the sample. For this study, the value of $I_0G.\varepsilon$ has been evaluated by separately running the K X-ray spectra of a number of spectroscopically pure thin foils (K, Ca, Sc, Ti, V, Mn, Fe, Co, Ni, Zn, Ge, Se, Rb, Sr, Y and Mo) of known concentrations (Micromatter, Deer Harbor, WA, USA) and using the expression:

$$I_0G.\varepsilon = \frac{n_{K_\alpha(j)}}{\sigma_{K_\alpha(j)}\beta_{K_\alpha(j)}m_j} \qquad (2)$$

where all the terms in equation (2) have the same meaning as in equation (1) except that they correspond to the K_α X-rays of a given element instead of ith group of X-rays. The weighted average of $I_0G.\varepsilon$ values obtained from different foils was plotted as a function of energy and is given in Figure 2. The solid line represents the least squares curve fitted to the experimental points. The $I_0G.\varepsilon$ values at various energies were calculated from the coefficients obtained from least-squares fitting. The error in $I_0G.\varepsilon$ value is estimated to be less than 3%.

The absorption correction factor (β) is given by the expression

$$\beta = \frac{1 - \exp[-(\mu_1/\cos\theta_1 + \mu_2/\cos\theta_2)t]}{(\mu_1/\cos\theta_1 + \mu_2/\cos\theta_2)t} \qquad (3)$$

where μ_1 and μ_2 are the mass absorption coefficients ($cm^2\,g^{-1}$) of the sample for the excitation and characteristic radiations respectively, θ_1 and θ_2 are the angles formed by the exciting and characteristic radiations respectively and t is the thickness of the sample in $g\,cm^{-2}$.

The values of β can be determined directly from equation (3) using the values of μ_1, μ_2, θ_1, θ_2 and t, but this is only possible for a known matrix.

In the present study, since the matrix was unknown determination of β was made by the experimental method (Kumar *et al.* 1989). In this method the value of β can be obtained by measuring the relative intensity of X-rays with and without the specimen from a target located at a position adjacent to the back of the specimen. The β values have been calculated separately for control and treated samples because of matrix variations.

Results

Typical fluorescence X-ray spectra of liver samples from control and lead-treated animals are shown in Figure 3a & b. X-ray peaks for K, Fe, Cu, Zn, Br and Rb are clearly visible (Pb was not detected in control liver samples). The concentrations of these elements have been calculated by evaluating the areas under various X-ray peaks and using

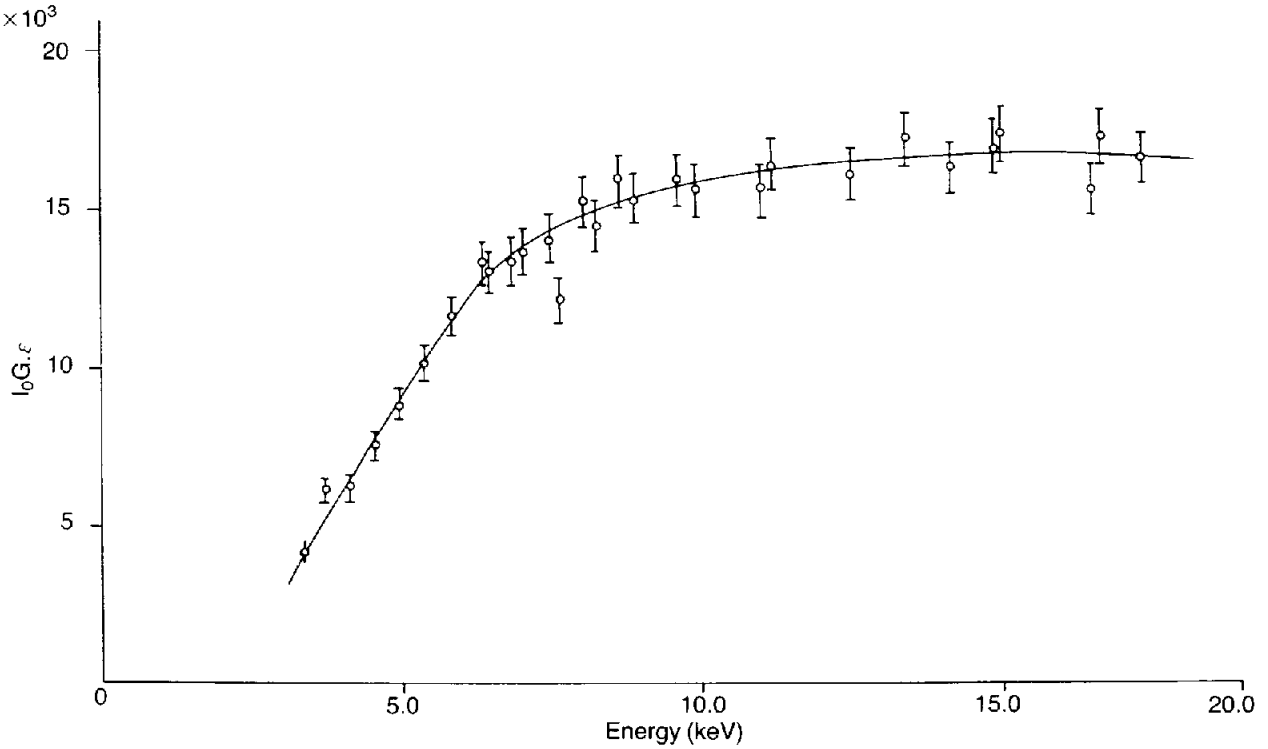

Figure 2. Plot of $I_0G.\varepsilon$ as a function of energy (keV). I_0G is the intensity of the exciting radiations and ε is the detector efficiency.

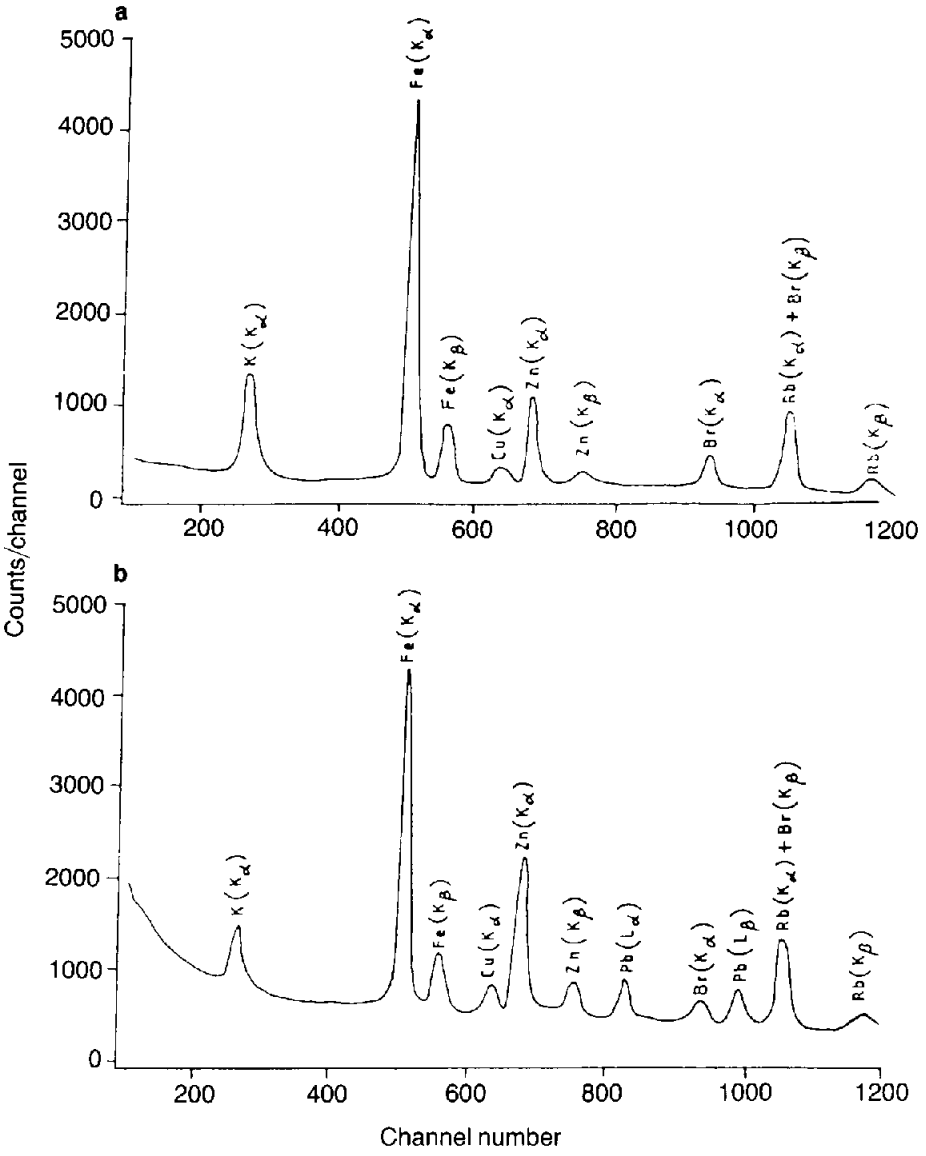

Figure 3. Typical X-ray spectra of liver samples from **a** control group and **b** lead-treated group, recorded with Si(Li) detector using ^{109}Cd exciter.

the fundamental parameter approach (Gedcke *et al.* 1982, Kumar *et al.* 1989).

The hepatic levels of various elements detected in the different groups of rats after short- and long-term treatments are presented in Table 1 (Pb was not detected in control liver samples). The administration of lead acetate (both low and high doses) significantly increased the levels of hepatic Fe after both short- and long-term treatments. However, the increase was more pronounced following long-term treatment. In contrast, the levels of K, Cu and Br decreased significantly with the administration of low and high doses of lead acetate for short and long periods. The decrease in K levels was most pronounced, decreasing by 40 and 59% with low and high lead doses, respectively. The hepatic Rb contents decreased significantly (11.5%, $P < 0.1$) only with low doses of lead for short duration. In contrast, the hepatic levels of Rb following long-term lead administration with low and high doses increased by 12 and 15% ($P < 0.1$) respectively. No significant change was observed in hepatic Zn contents after short-term lead treatment at either dose level, although long-term treatment resulted in significant increases of about 16% (low dose) and 23% (high dose).

Discussion

Fe

In our study lead supplementation to rats resulted in a significant increase in liver Fe contents, which was more pronounced after long-term treatment. We have previously reported (Singh *et al.* 1994) significantly increased levels of blood Fe in lead battery manufacturers. Kumar *et al.* (1991) have shown that Fe content of liver was raised by about 60% in animals exposed to automobile exhaust, and suggested that host defence response is enhanced so as to

Table 1. Elemental concentrations (per g dry weight) in liver samples of control rats and in rats treated with 50 or 100 mg lead/kg body wt after 1 and 4 months of treatment (values are means ± SD for 6–8 liver samples)

	Elemental concentration (% variation from control)						
	[mg/g]		[μg/g]				
	K	Fe	Cu	Zn	Br	Rb	Pb
Control	38.4 ± 5.0	0.34 ± 0.04	21.2 ± 2.5	94.7 ± 7.0	11.6 ± 2.6	28.8 ± 4.3	N.D.
1 month							
50 mg/kg*	31.9 ± 3.1**** (−17%)	0.47 ± 0.10*** (+38%)	15.4 ± 2.2***** (−27%)	90.0 ± 13.0 (−5%)	9.20 ± 1.7*** (−21%)	25.5 ± 3.3*** (−12%)	17.2 ± 3.5 –
100 mg/kg	33.7 ± 3.2*** (−12%)	0.50 ± 0.10***** (+47%)	15.0 ± 3.2**** (−29%)	86.6 ± 17.0 (+9%)	8.90 ± 2.1** (−23%)	27.1 ± 3.7 (−6%)	22.3 ± 7.5 –
4 months							
50 mg/kg	23.2 ± 4.3***** (−39%)	0.75 ± 0.34**** (+120%)	16.5 ± 2.2***** (−22%)	109 ± 13.0**** (+15%)	8.9 ± 1.1*** (−23%)	32.3 ± 3.9*** (+12%)	17.4 ± 2.0 –
100 mg/kg	15.8 ± 5.0***** (−59%)	0.95 ± 0.20***** (+179%)	14.7 ± 1.1***** (−31%)	116 ± 18.0**** (+23%)	9.1 ± 2.5* (−22%)	33.2 ± 5.1* (+15%)	21.9 ± 1.9 –

*$P < 0.1$; **$P < 0.05$; ***$P < 0.025$; ****$P < 0.01$; *****$P < 0.005$.
N.D. = not determined.

minimize the toxic effects of lead, thereby leading to the raised levels of hepatic Fe. It is thus speculated that the host defence system gets more activated with the increase in liver lead burden, leading to further enhancement in hepatic Fe content in order to combat the toxic conditions created by lead administration.

Cu

Our study has shown a significant decrease in hepatic Cu levels with the administration of low and high doses of lead to rats for both treatment durations. These findings are in agreement with the earlier observations of Klauder *et al.* (1972) who reported that dietary lead could reduce plasma Cu and ceruloplasmin levels effectively. An earlier report from our laboratory of a decrease of about 16% in the hepatic Cu contents of experimental rats exposed to automobile exhaust also supports the present study (Singh *et al.* 1994).

Zn

We have observed a significant increase in hepatic Zn levels following long- and short-term lead treatment (low and high doses). Lead has been reported (Eaton *et al.* 1980) to significantly induce metallothionein (MT) levels. Therefore, induction of MT by lead presumably results in enhanced concentration of Zn in liver. This increase in hepatic Zn content after lead administration is observed to be time-dependent since the increase in Zn content is more pronounced after long-term than short-term treatment. Lead has also been shown to inhibit δ-ALAD, although the activity of this enzyme is restored following zinc administration (Finelli *et al.* 1975). Thus, it may be speculated that the raised levels of Zn are attributable to its increased absorption through the gut, thereby neutralizing δ-ALAD and minimizing the toxic effects of lead. Further, Cerklewski & Fobes (1976) hypothesized that Zn and Pb compete for similar binding sites on a MT-like protein responsible for metal transport in the intestine. Interactions between Zn and Pb for common MT are possible beyond the level of GI tract. Thus, the competition between Pb and Zn for similar binding sites would possibly lead to increased hepatic Zn contents with more lead burden.

K

Lead administration (low and high doses) significantly decreased the K levels after short- and long-term treatments, however the decrease was more pronounced after treatment for long durations. Kumar *et al.* (1991) recently reported a significant reduction of about 12% in hepatic K levels of experimental animals exposed to automobile exhaust. Lead has been reported to alter membrane permeability, bind to active sites involved in K permeability and inhibit active transport by blocking Na^+/K^+ ATPase activity (Lakshmi 1989). Passow *et al.* (1961) reported that lead liberates normally inactive carrier molecules which are highly specific with respect to K and Rb; these molecules would in turn account for the counter-transport.

Rb and Br

Although non-essential, these two elements were detected in all the liver samples. They possibly enter the animal system from the environment, particularly through the diet. Kumar *et al.* (1991) found that when experimental rats were exposed to motor exhaust for 24 min per day for 3 months, a significant decrease of 13% was observed in hepatic Rb levels. We observed a similarly significant decrease ($P < 0.1$) in hepatic Rb levels following short-term low dose lead administration, although the long-term lead supplementation (at both dose levels) resulted in a

significant increase in hepatic Rb levels. In a recent report from our laboratory (Singh *et al.* 1994) we have shown increases of 27 and 45% in the blood Rb levels of automobile workers and lead battery manufacturers respectively. These workers were continuously exposed to lead and had spent 3–10 years in their occupational lead environments. We suggested that the levels of K in liver are decreased following lead treatment because of the liberation of inactive carrier molecules. Hence, the permeability changes resulting from alteration in Na^+/K^+ ATPase activity would possibly be restored by increase in the hepatic Rb contents.

The Br contents of liver were lowered significantly following short- and long-term lead treatment (low and high doses). Earlier reports (Kumar *et al.* 1991, Singh *et al.* 1994) have shown that human blood and experimental rat liver Br levels were higher when the people or rats had been exposed to automobile exhaust. Also, it has been reported (Hirschler *et al.* 1957, Habibi 1973) that tetra alkyl lead which is added to petrol as an antiknock agent reacts with ethylene dihalide and results in emission of lead along with Br and Cl in an inorganic particulate form. Therefore, the higher amounts of Br detected in human blood and rat liver samples exposed to automobile exhaust are possibly due to inhalation of the Br (HBr) produced during petrol combustion. In the present study, we have seen reduction in hepatic Br levels following lead treatment, which is possibly due to increased mobilization of hepatic Br stores.

Acknowledgments

This work was undertaken with the financial assistance of the Council of Scientific and Industrial Research, Government of India and the Indo-US foundation (PL-480).

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