Immunoaffinity-Assisted Purification of Tetrachlorodibenzo-*p*-dioxin in Milk

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Introduction

Polychlorodibenzo-*p*-dioxins are environmental contaminants which pose a health concern. Most human exposure is through food consumption, with milk accounting for nearly 50% of the dioxin intake[1]. Current analysis techniques for dioxins samples are costly[2], which is impractical for regulatory purposes as it would be financially prohibitive to analyze an appropriate number of samples.

Current dioxin analysis involves extensive sample cleanup prior to high resolution GC-MS. The typical cleanup procedure involves liquid-liquid extraction, followed by separation on multiple columns. Even with an automatic FMS Dioxin-Prep™ sample cleanup system (Fluid Management System, Watertown, MA) it is still labor intensive, and a laboratory with two technicians can typically perform ten samples per week[3]. Also, with this procedure, organic solvent waste including 1,840 ml of hexane, 265 ml of dichloromethane, 250 ml of toluene, 70 ml of ethyl acetate, and 70 ml of benzene per sample is generated causing additional environmental and health hazard concerns.

Antibodies have a variety of applications because of their specific binding recognition. Immunoaffinity columns (IAC) have been used as tools to separate target compounds from complex biological matrices[4,5], food products[6,7], and environmental samples[8]. An immunoaffinity column has been generated for 1,3,7,8-tetrachlorodibenzo-*p*-dioxin (1,3,7,8-TCDD). We present here the sample preparation needed prior to the immunoaffinity column, the elution conditions, and post immunoaffinity column cleanup procedures for the analysis of milk samples.

Experimental Methods

Sample Preparation Bovine milk was obtained from the North Dakota State University Dairy Farm, and samples were frozen and stored at -20 °C until used. In a glass vial 10 ml of milk was spiked with 1,3,7,8-Tetrachloro[7,8-dichlorophenyl-U-^{14}C]dibenzodioxin (specific activity 63.4 mCi/mmole, ChemSyn Science Laboratories, Lenexa, KS). The spiked samples were either applied directly to the IAC or applied after dilution with 1:20 or 1:50 0.05% v/v Triton X-100 (Bio-Rad Laboratories, Richmond, CA). An alternate procedure was to extract the milk sample with ethanol/hexane in a 1:2 ratio. The hexane fraction was passed through a 0.25 g graphite carbon solid phase extraction column (Carbograph, Alltech, Deerfield, WI). After washing the column with hexane and dichloromethane, the sample was eluted with toluene. The toluene was evaporated under a stream of nitrogen. The sample was resuspended in 15 ml of 0.05% v/v Triton X-100 and sonicated for 30 min before application to the immunoaffinity column.

Immunoaffinity Column Purification Antibodies generated from a 1,3,7,8-TCDD-keyhole limpet hemocyanin conjugate were used for coupling to CNBr-Sepharose (Pharmacia Biotech, Uppsala, Sweden)[9]. Ten ml of gel beads were packed into a glass column. The immunoaffinity column was equilibrated with 0.05% v/v Triton X-100 after which the processed milk sample was applied to the column. The column was washed with an additional portion of 0.05% v/v Triton X-100. Dioxin was eluted from the immunoaffinity column by increasing the Triton X-100 concentration to 0.5% v/v. The columns were stored in 10 mM phosphate buffer (pH 7.4) in the presence of 0.02% w/v sodium azide at 4 °C. The recovery of radioactivity was determined by liquid scintillation counting.

GC-MS analysis: The material eluted with 0.5% v/v Trition X-100 was extracted with ethanol/hexane. The hexane extract was passed through a 2 ml sintered glass funnel packed with acid silica[10]. The eluant was evaporated under a stream of nitrogen with 10 µl dodecane as a keeper and the solution analyzed by GC-MS. The GC-MS incorporated a HP 5890 series II GC (Hewlett-Packard co., San Fernando, CA) with a VG Autospec mass spectrometer (Micromass Inc., Beverly, MA) using a 15 m DB-5MS column, 0.25 mm id, 0.25 µm film thickness (J & W Scientific, Folsom, CA). The temperature program was: initial temperature 150°C for 2 min, ramped to final temperature of 310°C at 5 °C/min, and maintained at 310 °C for 4 min. A molecular ion (322) trace was selectively extracted from the data to monitor for 1,3,7,8-TCDD.

Results and Discussion

When the spiked milk was applied directly to the IAC, the column plugged. Resuspension of the immunoaffinity gel and washing resulted in removal of most of the radioactivity (Table I) indicating that the sample did not bind to the IAC. Diluting the milk sample increased the binding efficiency, and the column did not plug so that washing and elution could be carried out using the standard procedure. Increasing the dilution to 1:50, decreased the sample lost in the wash and increased the recovery of the sample in the elution step. However at even a 1:50 dilution, nearly one third of the sample did not bind to the column. Apparently milk contains constituents which interfere with the binding of the 1,3,7,8-TCDD with immunobinding sites of the column. Consequently a pre-affinity column cleanup utilizing a liquid-liquid extraction with ethanol/hexane followed by a Carbograph solid phase extraction was used to separate lipids from the dioxin (recovery for both extraction and Carbograph steps was 87.6 ± 17.4%). The binding efficiency improved dramatically using this cleanup protocol. Table I summarizes the application of dioxin to the immunoaffinity column and its pattern of recovery.

Table I. Application of dioxin to immunoaffinity column

dioxin (ng/ml gel)	n	application matrix	% in wash	% in eluant
12.5	1	undiluted milk	92.6	3.4
15.7	1	milk 1:20	49.9	27.9
13.9	1	milk 1:50	32.5	55.4
13.1 ± 0.9	3	EtOH/Hexane extract and Carbograph SFE	5.7 ± 7.3	86.9 ± 5.4

For post-affinity column cleanup, both ethanol/hexane extraction and acid silica column steps were necessary to remove the Triton X-100 which interfered with GC-MS analysis (recovery for both steps was 79%). The GC-MS selective ion trace showed 1,3,7,8-TCDD had a retention time of 13:12 min.

In conclusion, the immunoaffinity column provided specificity, but required a pre-column cleanup of the milk prior to IAC. Dilution of milk samples alone improved the operation of the IAC but did not give sufficiently high recovery for practical analysis. Using this approach, it should be possible to decrease organic solvent usage and the steps needed for dioxin sample cleanup prior to GC-MS analysis. Currently, IAC analysis of one sample uses 20 ml of ethanol, 60 ml of hexane, 20 ml of dichloromethane, and 20 ml of toluene. Optimization of the cleanup procedure to further decrease organic solvent usage is under investigation.

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