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Project Summary

Biological Monitoring Systems for Hazardous Waste Sites (Production and Analysis of Analytical Reference Materials)

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Portions of EPA programs in pesticides, toxics, and hazardous waste have a need for various types of analytical reference material. The current project has emphasized the collection and analysis of urine, fat, and blood from cattle exposed to selected toxicants for ultimate use as reference samples. The project also addressed the practicality of using certain metabolites produced by the cattle to indicate previous exposure to chlorinated hydrocarbons and the likelihood that these metabolites could serve as conditions of exposure even if an animal had ingested small amounts of many other chemicals such as might be present in the vicinity of an uncontrolled hazardous waste site.

The reference samples of urine, fat, and other tissues can, with verified compound concentrations, ultimately be used as qualifying samples when selecting an analytical laboratory from among several candidates or when selecting the best technique to use for a particular analysis. However, the reference materials may be of greatest benefit when used by laboratories to determine analytical accuracy for samples of human urine, blood. etc. This is because the standards, like the unknown samples, will contain pollutant compounds and associated metabolites (all *in vivo* incorporated) in an appropriate biological matrix.

Dairy animals were used during this study primarily because substantial quantities of tissues and body fluids could be collected from the previously dosed cattle. While none of the individual study phases included a thorough evaluation of dosing compound retention, excretion, and/or metabolic degredation, each animal was given daily oral doses of selected toxicants so that the urine, blood, etc., would contain detectable concentrations of the dosing compound(s) and some of the associated major metabolites. Aliquots of the respective large-volume samples were chemically analyzed and, based on the initial analytical results, some of the samples were then selected as potential reference materials. Several confirming analyses (not part of this specific project) will ultimately be conducted before any sample is actually distributed as an analytical reference or quality assurance material.

The project report contains several data summaries organized by individual animal and by individual dosing compound. Analytical and sample preparation steps are discussed and the analytical detection limits are included for each dosing compound and for each sample type. Some of

the samples provided by this study have already been used in an ongoing EMSL-LV program for analytical method optimization.

This Project Summary was developed by EPA's Environmental Monitoring Systems Laboratory, Las Vegas, NV, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Assessing current and potential problems at uncontrolled hazardous waste sites has been very difficult due to several factors including (1) the complex chemical mixtures found at most sites and (2) the fact that toxicity and environmental transport data are limited for many of the compounds present at the site, especially for those that are by-products of organic synthesis (rather than commercial chemicals). Similarly, when samples of urine, blood, milk, etc., are collected and analyzed in an attempt to assess exposure and uptake, it is very difficult to determine the accuracy of these analyses due to a lack of adequate reference materials. Under conditions of mammalian exposure, many of the pollutants would be partially metabolized and several conjugation or degradation products would be present in a collected sample. The currently available standards are usually prepared by adding the pollutant compound(s) to an uncontaminated sample of biological tissues or fluids. While this approach provides a reference compound in the correct sample matrix, it does not include the array of associated compound degradation products, many of which can interfere with an accurate analytical determination and some of which may have high toxicological significance. Portions of EPA programs in pesticides, toxics, and hazardous waste have a defined need for analytical reference and quality assurance materials.

This study was conducted to determine (confirm) the retention and excretion pattern of selected hazardous waste site chemicals and to provide analytical reference and quality assurance samples that contain both a representative concentration of the pollutant compound(s) and representative concentrations of the associated metabolic degradation products. Cattle were used as experimental animals throughout the study (1) because of their potential selection as a biological monitoring species for chemical pollutants in agricultural areas, (2) because they provide major food products for human consumption (i.e., milk and beef) and, perhaps most importantly, (3) because substantial quantities of mammalian tissue and body fluid could be collected and prepared for subsequent use as reference materials. In each phase of the project, cattle were given daily oral doses of compounds that are of regulatory interest to one or more of the EPA program offices. Samples of urine, blood, milk, feces, and sacrifice tissues were subsequently collected and portions of the respective samples were analyzed using computer assisted gas chromatography/ mass spectrometry. The selection of chlorinated hydrocarbons, emphasized in phases I and II, was based on results from a 1980 Love Canal (Niagara Falls, New York) monitoring program and on a preliminary survey of toxic chemicals frequently present in hazardous wastes.

During the first part of this project, some brief method evaluation tests were conducted to select the specific analytical and sample preparation procedures that would be used during subsequent study phases. Another objective of the initial effort concerned the practicality of using certain metabolites to indicate previous exposure to chlorinated hydrocarbons and whether these metabolites could be used if an animal had ingested not only chlorinated hydrocarbons but had also ingested small amounts of many other waste site chemicals. Indicator compounds would include those compounds and metabolites that, when detected in urine, blood, etc., suggest previous exposure to chlorinated hydrocarbons.

The second part of the overall project (and the phase for which there is a continuing EPA program office interest) emphasized the collection and analysis of blood, fat, and urine samples for ultimate use as analytical reference materials. Substantial quantities of body fluid were easily obtained from the dairy animals. Each animal was given daily oral doses of selected pollutant chemicals so that the tissues and fluids, would contain detectable concentrations of the dosing compound(s) or associated major metabolites. Aliquots of the respective large volume samples were chemically analyzed and, based on these initial analytical results, some of the samples were then selected as potential reference samples. Confirming chemical analyses will ultimately be conducted before final sample selections are made and before any sample aliquots are distributed as reference or quality assurance materials.

The reference samples, with ver compound concentrations, can be as performance evaluation samples v selecting an analytical laboratory among several candidates or w selecting the best technique to use f particular analysis. However, the re ence materials may perhaps be greatest benefit when used by lab tories to determine analytical accu for samples of human urine, blood, As mentioned previously, this is bec the standards, like the unknown samp will contain pollutant compounds associated metabolites (all *in vivo* in porated) in an appropriate biolog matrix.

Sample Collection

Dosing groups for the four indivic study phases are shown in Tables 1, 2 and 4, respectively. No dosing adj ments were made for individual variati in animal weight. However, individ animal weights were taken before dos began and at time of sacrifice. All anim received a commercial feed preparat and had continuous access to water fr individual automatic watering units.

In phase I, dairy cows were ora dosed with a mixture of chlorinat hydrocarbons (2,4-dichlorophenol, 1 dichlorobenzene, lindane, 1,2,3,4-tet chlorobenzene, and pentachlorophen Selected tissues (e.g., liver, kidne muscle, fat, and blood), milk, urine, a feces were analyzed for both the par dosing compounds and for some of t major metabolic conjugation/ degradati products. During phase II, eight anim received various combinations of tl phase I chlorinated hydrocarbons and liquid dose that had originally be collected from an actual hazardous was site. While the waste site liquid was n toxic at the amounts administered, it d contain a broad array of organic cor pounds. Large volume samples of urir were retained for possible use a analytical references and, as in phase all collected sample types were analyze for the chlorinated hydrocarbon dosir compounds and for some of the maj metabolic degradation products.

Phase III and phase IV were conducte strictly to provide samples for ultimat use as analytical reference and qualit assurance materials and, in some ir stances, to provide sample material fc use in a continuing EMSL-LV program c analytical method optimization. The diver sified group of phase III and phase I dosing chemicals included a carbamat insecticide, some polycyclic aromati

Table 1. *Chlorinated Hydrocarbon Doses Administered to Phase I Dairy Cattle*

Dosing Groups	Dose Per Cow: Daily dose per animal	Dose Per Cow: Total dose for 28 days	Dose Per Cow: mg/kg/day (approximate)
Two Animals (Group I)			
lindane	*4.0 grams*	*112 grams*	*6 mg/kg*
Two Animals (Group II)			
lindane	*2.0 grams*	*56 grams*	*3 mg/kg*
1,2-dichlorobenzene	*20.0 grams*	*560 grams*	*29 mg/kg*
2,4-dichlorophenol	*20.0 grams*	*560 grams*	*29 mg/kg*
1,2,3,4-tetrachlorobenzene	*2.0 grams*	*56 grams*	*2 mg/kg*
Two Animals (Group III)			
lindane	*1.0 gram*	*28 grams*	*2 mg/kg*
1,2-dichlorobenzene	*20.0 grams*	*560 grams*	*29 mg/kg*
2,4-dichlorophenol	*20.0 grams*	*560 grams*	*29 mg/kg*
1,2,3,4-tetrachlorobenzene	*2.0 grams*	*56 grams*	*3 mg/kg*
pentachlorophenol	*1.0 gram*	*28 grams*	*2 mg/kg*

Note: Samples from this phase have already been used as part of the EMSL-LV program for analytical method optimization (Marsden, P.J., E.N. Amick, F.L. Shore, L.R. Williams, V.R. Bohman, and C.R. Blincoe. 1986. Characterization of Bovine Urine and Adipose Interlaboratory Performance Evaluation Samples Containing Biologically Incorporated Chlorophenols. Journal of Agriculture and Food Chemistry. 34:795-800)

hydrocarbons, an ether, a phthalate, an azobenzene dye, and an amine. Large volume samples were again collected from the animals, but at less frequent intervals than in phase I and II. In addition, less attention was given to the identification of specific metabolic products that resulted from the respective dosing compounds and, since the materials of primary interest were urine, blood and carcass fat, fewer types of samples were collected.

Throughout the project, samples were placed in Teflon containers and stored at -23°C. Blood samples were collected by jugular venipuncture using a 14 gauge syringe needle which allowed the blood to drain directly into the Teflon sample container. Portable machine milkers were routinely used during those times when the milk collection was not retained for chemical analysis. When milk samples were collected for analysis, the animals were milked by hand so that the milkwent directly into the sample collection container. Urine samples were typically taken before dosing began and for the first week of dosing. In some instances, the cows were catheterized within dwelling, inflatable urinary catheters and the urine would drain through polyethylene tubing into Teflon containers located at the rear of each stall. When tissue samples were collected, the animals were sacrificed two days after the last dose.

Sample Analysis

A continuing, though secondary, objective of this project was to improve the applicability of currently available procedures for the more difficult sample types, i.e., those with a significant lipid content such as milk, milk fat, carcass fat, and liver. Some of the extraction steps were modified primarily during the second phase of the study, and additional procedural revisions were made when analyzing for the more diversified group of chemicals present in the phase III and phase IV samples.

The amount of sample, or starting material, used during an analysis was typically 200 grams for urine and milk: 50 grams each for liver, muscle, kidney, and blood: 10 grams for fat: and, 100 grams for feces and animal feed. The sample analysis plan typically included homogenization of the sample material, enzymatic hydrolysis, solvent extraction, cleanup and concentration of solvent extracts, extract derivatization, and instrument analysis. Samples of animal tissue, feed, and feces were initially homogenized using a tissue grinder. With the exception of carcass fat and animal feed samples, the sample material was frequently treated with a commercial enzyme preparation to hydrolyze glucuronide and sulfate conjugation products. The hydrolyzed samples were then extracted with organic solvents at high and low pH to partition the base- neutral and acidic compounds. Extracts were then concentrated using either a Buchi rotoevaporator or a Kuderna-Danish apparatus. The acid fraction was then derivatized to improve the stability of free hydroxy compounds (i.e., compounds resulting from the derivatization step). The sample extracts were then analyzed using computer assisted gas chromatography/mass spectrometry. The approximate detection limits that were achieved during the study are shown in Table 5 and a precision estimate, based on the analysis of 10 aliquots of a single urine collection, is shown in Table 6.

The specific analytical and sample preparation steps that were used during each phase of the study are included in the project report. Dosing compounds and a few of the metabolite compounds (those that were commercially available) were added to some of the samples in order to allow for a subsequent estimate of compound recovery. The resulting compound recovery data are also provided in the project report.

Confirming Analysis

This study has provided many large volume samples and aliquots of these samples have received an initial analysis. Consequently, some of the samples have been tentatively selected as potential reference material and the remaining samples, collected as part of this overall effort, have been discarded. Carcass fat, blood, and urine represent the predominant number of sample types retained.

The next step will be to assess the stability of compound concentrations under typical storage conditions. This is obviously a critical area and analyses will be conducted at various intervals of time and, perhaps, under different storage temperatures. Another series of analyses is planned to assess the variability in compound concentration between aliquots of the same sample. The samples would not necessarily serve as a reference material for each *in vivo* incorporated pollutant compound and/or associated metabolite that might be present in a given sample, but rather would be used for one or more of the compounds where extensive confirmation analyses have been conducted. These confirming analyses are frequently conducted by more than one analytical technique.

Conclusions

None of the study phases was conducted as typical metabolism experi-

Table 2. *Phase II Cattle that Received Doses of an Actual Hazardous Waste Site Liquid as well as the Previously Administered Chlorinated Hydrocarbons*

Number of Animals	Chlorinated Hydrocarbon Dose*	Liquid Material from Hazardous Waste Site**	
		Dose/Animal/Day	Total Dose/Animal
2	tratment A	80 ml	1120 ml***
2	treatment A	160 ml	2240 ml***
2	1/2 tratment A	160 ml	2240 ml***
2		80 ml	2240 ml****
2			

*Chlorinated hydrocarbon dose (treatment A) composed of lindane - 1 gram/animal/day; 1,2-dichlorobenzene - 20 grams/animal/day; 2,4-dichlorophenol - 20 grams/animal/day; 1,2,3,4-tetrachlorobenzene - 2 grams/animal/days and pentachlorophenol - 1 gram/ animal/day.

**Waste site material contained many semivolatile organics at mg/l to µg/l concentrations, i.e., benzaldehyde; benzoic acid; pentachlorophenol; 9,10-anthracenedione; flouranthene; pyrene; p-phenylcarbanilic acid; 1-methoxy-1-methylethoxy-2-propanol; 4-methoxy 2,2,6-trimethyl cyclohexanone; 4-hydroxy-3-methoxy-benzaldehyde; phenanthrene; anthracene; carbazole; biphenylene; 4-methylphenol; 2-butoxyethanol; flourene; 1,2-benzenedicarboxylic acid, 1-ethoxybutane; trans-2-chlorocyclohexanol; phenoxyacetic acid: 4-hydroxybenzene acetic acid; methylcycloheptane; 5-methyl-1,2-haxadiene; 3-ethyl-1,4-hexadiene; phenanthridiene; phenol: 2,3,4,6-tetrachlorophenol; 2-methylanthracene; dibenzofuran; and N-acetylbenzamide. Numerous inorganics (mg/l concentrations) were also present, i.e, aluminum - 420; cadmium - 14; calcium - 200; chromium - 440; cobalt - 130; copper - 710; iron - 470; lead - 3; magnesium - 690; manganese - 38; molybdenum - 21; nickel - 130; thallium - 20; and zinc - 48.

***Administered by rumen catheter (material given last 14 consecutive days, i.e., day 14-28)

****Administered by rumen catheter (material given for 28 consecutive days)

ments where all major metabolic degradation products are identified and their retention and excretion quantitated. However, various data summaries are included in the project report that present individual compound concentrations for each animal and for each tissue or body fluid. Some progress was also made with the analysis of milk fat and carcass fat samples where most of the initial analytical difficulty was encountered. The analytical procedures are also thoroughly addressed in the project report.

Mammalian patterns of chlorinated hydrocarbon metabolism suggested that the five compounds administered in phases I and II, would yield several degradation products, e.g., 3,4-dichlorophenol; 2,3-dichlorophenol; 2,4-dichlorophenol; 2,3,5-trichlorophenol; 2,4,5-trichlorophenol; 2,3,4.6-tetrachlorophenol; 2,3,4,5-tetrachlorophenol; 2,4-dichlorophenylmercapturic acid; 3,4-dichlorophenylmercapturic acid; pentachlorooyclohexanol; 3,4-dichlorocatechol; 4,5-dichlorocatechol; and tetrachlorohydroquinone. Several of these compounds were in fact noted in the urine samples collected during this study. If animals were used as biological monitors for these waste site chemicals, the most likely indicator compounds (i.e., compounds that indicate previous exposure to a given group of chemicals) would probably be the presence of various phenols in the urine and long-half-life lipophilic compounds in the carcass fat.

The specific samples collected during this project may, or may not, be distributed as actual reference materials depending upon user needs. However, the analytical procedures and the sequence of confirming analyses (necessary for sample verification) have been established or improved during the various phases of this project. Large volume samples with *in vivo* incorporate toxicants can be provided in subseque efforts if additional material is needed f actual distribution. The reference ar quality assurance samples are intende for use as performance evaluation mate ials for use in selecting an analytic laboratory from among several cand dates or when selecting the best tecl nique to use for a particular analysis. Th EMSL-LV has already used the carcas fat and urine samples in its ongoin analytical method development an optimization program. Many laboratorie routinely analyze human urine sample and the use of these reference material should improve the accuracy of thes determinations. Difficulties in obtainin "safe" human blood in bulk quantities and in finding laboratories willing to wor with it, provides additional interest in th use of bovine blood for method an laboratory performance evaluations.

Table 3. *Dosing Regime and Sample Collection Schedule for Phase III Cattle*

	Compound	Hereford Heifers: Daily dose per animal (grams)	Hereford Heifers: Total dose per animal (grams)	Adult Holstein Cows: Daily Dose per animal (grams)	Adult Holstein Cows: Total dose per animal (grams)	Samples Collected: Urine	Milk	Blood
Pretreatment						x	x	x
Week 1	hexachlorobenzene	2.5	(see	5	(see			
	yellow dye no. 3	2.5	final	5	final			
	dioctyl phthalate	5.0	total	10	total			
	ethoxyethyl acetate	10.0	below)	20	below)	x	x	x
Week 1	hexachlorobenzene	2.5		5				
	yellow dye no. 3	2.5		5				
	dioctyl phthalate	5.0		10				
	polychlorinated biphenyls	5.0		10		x	x	x
Week 1	hexachlorobenzene	2.5		5				
	yellow dye no. 3	2.5		5				
	dioctyl phthalate	5.0		10				
	methylene dianiline	2.5		5				
	polybrominated biphenyls	2.5		5				x
Week 1	hexachlorobenzene	2.5	70	5	140			
	yellow dye no. 3	2.5	70	5	140			
	dioctyl phthalate	10.0	175	20	350			
	ethoxyethyl acetate		40		80			
	polychlorinated biphenyls		40		80			
	methylene dianiline	2.5	35	5	70			
	polybrominated biphenyls	2.5	33	5	65			
	carbaryl	2.5	18	5	35	x	x	x
	pentachlorophenol	0.5	4	1	7			

Note: *Ethoxyethyl acetate doses were discontinued after 4 days because of apparent toxicity. The PCB doses were administered until the supply of the PCB aroclor was exhausted (8 days). The PBB doses were not administered on the first day of week 4 due to a shortage of the PBB mixture.*

Table 4. *Compounds Given to Phase IV Dairy Cattle.*

Number of Animals	Compunds	Dose/Animal/Day	Total Dose/Animal
one	2-chlorodiphenyl ether	1 gram	21 grams
	hexachlorobenzene	1 gram	21 grams
	perylene	1 gram	21 grams
one	polychloronaphthalene no. 1099	1 gram	21 grams
	1,3,5-trichlorobenzene	1 gram	21 grams
	diethylhexyl adipate	1 gram	21 grams
one	polychloronaphthalene no. 1014	1 gram	21 grams
	n-dioctyl pthalate	1 gram	21 grams
	fluorene	1 gram	21 grams
one	fluoranthene	1 gram	21 grams
	arochlor 1260 (PCB)	1 gram	21 grams
	triphenyl phosphate	1 gram	21 grams

Note: *Solids were administered orally in gelatin capsules and liquids (diethylhexyl adipate, 2-chlorodiphenylether, and dioctyl pthalate) were infused through a rumen catheter. Blood serum, urine, carcass fat, and liver samples were collected from the animals for ultimate use as analytical reference materials.*

Table 5. *Estimate of Analytical Detection Limits Achieved During the Current Study when Analyzing the Various Types of Sample Material (Best Estimate Values Presented as ug/g of Sample Material)*

Compound	Detection Limits (µg/g) Achieved Using Different Sample Materials							
	Blood Serum	Milk	Milk Fat	Urine	Feces	Liver	Kidney	Carcass Fat
tetrachlorobenzene	0.01	0.01	ND	0.001	0.01	0.01	0.01	0.03
carbaryl	0.02	ND	0.05	0.01	ND	0.1	No	0.1
methylene dianiline	0.02	ND	0.5	0.01	ND	ND	ND	ND
dichlorophenols	0.01	0.01	ND	0.01	0.01	0.01	0.01	0.03
trichlorophenols	0.01	0.01	ND	0.01	0.03	0.03	0.02	0.1
tetrachlorobenzene	0.01	0.01	ND	0.001	0.01	0.01	0.01	0.03
dioctyl phthalate	0.01	ND	0.1	0.01	ND	0.20	ND	0 2
lindane	0.01	0.01	ND	0.01	0.05	0.03	0.03	0.01
yellow dye no.3	0.02	ND	1.0	0.01	ND	1.0	ND	1.0
polychlorinated biphenyls	0.02	ND	0.02	0.01	ND	0.5	ND	0.5
polybrominated biphenyls	0.05	ND	0.05	0.01	ND	0.5	ND	0.5
ethoxyethyl acetate	0.01	ND	ND	0.01	ND	ND	ND	ND
pentachlorophenol	0.02	0.01	0.2	0.01	0.05	1.0	0.03	1.0
dichlorobenzne	0.01	0.01	ND	0.001	0.01	0.01	0.01	0.03
hexachlorobenzene	0.5	ND	0.01	0.01	ND	0.1	ND	1.0

Note: *Varying amounts of analytical interference were typically encountered from sample to sample and the above values are presented as an approximation of the detection limits actually achieved when analyzing the different types of sample material. Carcass fat, milk, and milk fat were probably the most difficult of the samples to analyze. The notation ND (not determined) means that a detection limit estimate was not achieved when using the indicated sample material.*

Table 6. *Determination of Analytical Precision Based on Analysis of 10 Separate Aliquots of a Single Urine Sample. (Precision Expressed as Coefficient of Variation for 11 in vivo Incorporated Compounds Present in the Urine.)*

Compound	Number of Determinations	Mean Concentration (µg/g)	Coefficient of Variation (%)
tetrachlorobenzene	10	0.05	40
tetrachlorophenol	10	27.78	35
2,4,5-trichlorophenol	10	5.74	38
1,2-dichlorobenzene	10	0.13	15
3,4-dichlorophenol	10	7.07	44
2,3- and 2,4-dichlorophenol	10	8.27	33
lindane	10	0.19	37
phenol	10	3.39	44
methyl phenol	10	1.65	56
tetrachlorohydroquinone	10	2.42	44
pentachlorophenol	10	42.70	35

Note: *Ten aliquots of a single urine sample were taken and sequentially analyzed. Sample preparation steps, GC/MS analysis, and routine quality control steps were conducted for each aliquot.*

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The complete report, entitled "Biological Monitoring Systems for Hazardous Waste Sites (Production and Analysis of Analytical Reference Materials)," (Order No. PB 89-1[illegible]204/AS; Cost: $21.95, subject to change) will be available only from:

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