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DERMAL ABSORPTION AND TARGET ORGAN TOXICITY OF H-19457C HYDRAULIC FLUID

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Lt Col, USAF, BSC
Deputy Director, Toxicology Division
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Phosphate ester-based hydraulic fluids described by Military Specification H-19457 are being used in Navy hydraulic systems. Acute toxicity tests using this hydraulic fluid indicated little toxicity. Repeated inhalation exposures to several species of animals resulted in hepatotoxicity in rats only. Gavage studies with this fluid resulted in endocrine and reproductive effects after repeated doses of less than 2.0 g/kg. Because exposure to hydraulic fluids in the Naval environment often is by dermal contact, this study was designed to determine whether target organ effects found after oral administration could be reproduced by dermal administration of the hydraulic fluid. The initial phase of the study determined the bioavailability of the hydraulic fluid following single oral and dermal treatment. The hydraulic fluid was rapidly absorbed following either route of administration. Repeat dermal administration of the hydraulic fluid determined the ovary, adrenal, kidney, and liver as target organs. Serum estradiol levels were increased, however, estrous cycles were unaffected.

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PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Toxic Hazards Research Unit, ManTech Environmental Technology, Incorporated. This document serves as a final report on the dermal toxicity of H-19457C (Fyrquel 220®). The research described in this report began in August 1990 and was completed in April 1991 under U. S. Air Force Contract Nos. F33615-85-C-0532 and F33615-90-C-0532. LtCol James N. McDougal served as Contract Technical Monitor for the U. S. Air Force, Armstrong Laboratory. This study was sponsored by the U. S. Navy under the direction of CAPT David A. Macys, MSC, USN.

This work was supported by the Naval Medical Research and Development Command Task M0096.004.0006. The opinions contained herein are those of the authors and are not to be construed as official or reflecting the view of the Department of the Navy or the Naval Services at large.

The authors would like to thank Carlyle Flemming for statistical analysis. Acknowledgement is also made to Susan Dille, Richard Godfrey, and Janet Wilson for their excellent technical assistance.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ALT	Alanine aminotransferase
amu	Atomic mass unit
AST	Aspartate aminotransferase
C	Celsius
Col	Column
EDTA	Ethylenediaminetetraacetic acid
eV	Electron volts
F	Fahrenheit
F-344	Fischer 344 (rat)
g	Gram
GC	Gas chromatograph
GC/MS	Gas chromatograph/Mass spectrometer
h	Hour
kg	Kilogram
m	Meter
m^3	Meter cubed
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MS	Mass spectrophotometer
msec	Millisecond
m/z	Mass/charge
µg	Microgram

µL	Microliter
µm	Micrometer
N	Number
ng	Nanogram
NMRI/TD	Navy Medical Research Institute/Toxicology Detachment
NS	Not significant
p	Probability
pg	Picogram
Ret t	Retention time
sec	Second
SEM	Standard error of the mean
Temp	Temperature
THRU	Toxic Hazards Research Unit
v/v	Volume/volume
Wt	Weight
$\bar{x}$	Mean

SECTION 1

INTRODUCTION

Phosphate ester-based hydraulic fluids described by Military Specification H-19457 are used extensively in Navy hydraulic systems, including aircraft and weapons elevators. One of these hydraulic fluids, H-19457C (trade name, Fyrquel 220®, referred to as Fyrquel in this report), has been studied extensively in this laboratory. An acute battery of toxicologic screens, including neurotoxicity evaluation, found Fyrquel to be relatively nontoxic (Gaworski et al., 1986). Repeated inhalation exposures (21 days) to rats, hamsters, and rabbits at 250 mg/m^3 resulted in an increase in relative liver weights in rats only. Exposure to lower concentrations of 100 and 10 mg/m^3 for 90 days had similar effects on the liver as well as increased relative kidney weights in rats from the high-exposure group (Wall et al., 1990).

Gavage studies with Fyrquel have shown deleterious endocrine and reproductive effects in Fischer 344 (F-344) rats after repeated doses of 1.68 g/kg. A continuous breeding study to assess the reproductive toxicity of Fyrquel resulted in a decrease in fertility of breeding pairs and significantly reduced the mating and fertility indices of female rats. Fyrquel produced a treatment-related decrease in litters per fertile pair, depressed body weights, and increased relative liver weights. Other gavage studies with Fyrquel have shown increased estradiol levels, and changes in estrous cycles occurred as early as three weeks into the 9-week test period. Ovarian and adrenocortical lipidoses were noted at necropsy (Latendresse, unpublished data 1990).

Exposure to hydraulic fluids in the Naval environment often occurs from vapors or aerosols coming into direct contact with the skin. Skin contact also results from material handling, systems maintenance, and damage control operations. The Naval Medical Research Institute, Toxicology Detachment (NMRI/TD) requested that theToxic Hazards Research Unit (THRU) conduct studies to determine if the target organ effects, including estradiol increases caused by gavage, were achievable by dermal exposure.

It was the purpose of this study to develop data that will determine whether target organ effects found after oral administration can be reproduced by dermal administration of Fyrquel.

SECTION 2

MATERIALS AND METHODS

ANIMALS

Upon receipt, female F-344 rats (Phase I rats from Charles River, Kingston, NY; Phase II rats from Harlan Sprague-Dawley, Indianapolis, IN) were quality control tested and found to be in acceptable health. They were group housed (three per cage) in clear plastic cages with wood-chip bedding (Bettachip®, Northeastern Products Corp., Warrensburg, NY) prior to initiation of the study and singly housed in the same manner during the study. The rats were randomized using a proprietary modular software system (PATH/TOX® System, Xybion Medical Systems, Cedar Knolls, NJ) which assigned animals to groups by a stratified randomization procedure based on body weight. Water and feed (Purina Formulab #5008) were available ad libitum except for 12 h prior to sacrifice. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

TEST MATERIAL

Fyrquel is an isomeric mixture of butylated triphenyl phosphates. The major components of Fyrquel are *p*-*t*-butylphenyl diphenylphosphate (52.6%), bis(*p*-*t*-butylphenyl) phenylphosphate (30.2%), and triphenyl phosphate (13.2%). The test material was supplied by NMRI/TD, Wright-Patterson Air Force Base, OH. Lot number identification and physical and chemical properties of the hydraulic fluid follow.

Mil. Spec. No.:	MIL-H-19457C
Trade Name:	Fyrquel 220®
Manufacturer:	AKZO Chemicals, Inc.
Lot Number:	3820J-1
CAS Number:	115-86-6
Physical State:	Liquid
Sp. Gravity (60 °/60 °F):	1.150
Boiling Point (°F):	735
Odor:	Very slight
Viscosity (centistokes):	219

TREATMENT REGIMEN

Phase I

To determine the skin absorption of H-19457C and compare it with absorption following oral administration, the test material was administered neat at 1 68 g/kg body weight by either single gavage or single dermal administration Following treatment animals were sacrificed at 1, 4, 24, and 48 h Blood, muscle, liver, kidneys, skin, fat, adrenal, and ovary were sampled for Fyrquel analysis The remaining adrenal and ovary were removed for histopathologic examination The animals sacrificed at 24 h were housed in metabolism cages for collection of urine and fecal samples, which were also analyzed for Fyrquel

Phase II

To determine endocrine effects of repeated dermal application, the test material was administered at a dose of 1 68 g/kg body weight to the unmodified skin of the clipped backs of 15 rats, 6 h per day, on a daily basis for periods up to 6 weeks The test area was not occluded; however, collars were fitted on the rats during the 6-h treatment period to prevent oral ingestion of the test material The rats were weighed weekly and the dosage calculated for the following week A similar treatment, using sesame oil, was performed on a control group of 15 rats Daily vaginal washes were done on each rat to monitor estrous cycles Groups (5 test and 5 control) were sacrificed following 14, 28, and 42 days Adrenals, ovaries, kidneys, and livers were weighed at sacrifice To compare with results determined following gavage, terminal blood samples were taken for total protein, albumin, serum globulin, AST, and ALT measurements In addition, estradiol levels were determined Adrenals, ovaries, kidneys, thymus, and livers were sampled for histopathologic examination

ANALYSIS OF BIOLOGICAL SAMPLES

Physiological Sample Analysis

A method of analysis using combined gas chromatography/mass spectrometry (GC/MS) was developed to measure the major components of Fyrquel in physiological samples The method involved a solvent extraction step and GC/MS analysis with selected ion monitoring The chromatograph column was a 12-m x 0 2 mm HP1 column supplied by Hewlett-Packard Corp (Avondale, PA) Chromatographic separation was obtained using a Hewlett-Packard 5890 GC equipped with a Hewlett-Packard 7673A Autosampler The GC was interfaced to a Hewlett-Packard 5970 Mass Selective Detector (MSD) quadrupole mass spectrometer equipped with a 70 eV electron impact source The electron optics were calibrated using perfluorotri-*n*-butyl amine and mass spectra were obtained scanning from 50 to 700 atomic mass units (amu) Ions at mass charge

ratio (m/z) 326, 367, and 425 were monitored for triphenyl phosphate, *p*-*t*-butylphenyl diphenylphosphate, and bis(*p* *t* butylphenyl) phenylphosphate, respectively

A Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) was used for the homogenization of tissue samples and a Haake-Buchler vortex evaporator (Haake-Buchler, Inc., Saddlebrook, NJ) was used for the extraction of biological samples. Approximately 0.5-g samples of liver, kidney, fat, muscle, skin, and feces were placed in 20-mL scintillation vials and homogenized in 5 mL of 0.9% saline for 5 min. Urine samples were processed in this manner without homogenizing. Similarly, 0.1-g samples of adrenal and ovary were homogenized in 1.0 mL of 0.9% saline using 4-mL glass vials. Blood samples were collected in EDTA tubes. A 1.0-mL sample of whole blood was transferred to a 4-mL glass vial for extraction. All samples were extracted with a 1:1 (v/v) mixture of hexane-ethyl acetate. An internal standard, tri-*m*-tolyl phosphate, was added to the hexane-ethyl acetate layer. The samples were vortexed overnight, then centrifuged at 2000 x g for 20 min. The organic layer was removed for analysis. The chromatography conditions for analysis are listed in Table A of Appendix A. A more complete description of the GC/MS analysis of Fyrquel can be found in the chemistry report attached to this technical report (see Appendix A).

Estrous Cycle Monitoring

A daily vaginal saline wash (prior to 10:00 a.m.) was done on each rat to monitor estrous cycles. Slides were prepared and examined microscopically. Cytology slides were subsequently air dried, stained using Wright's stain, cover slipped, and archived.

Clinical Chemistry

Sera for clinical chemistry evaluations were assayed on an Ektachem 700 XR (Eastman Kodak, Rochester, NY). Serum estradiol was measured using a double antibody competitive binding radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA).

Histopathology Evaluation

Tissues for histopathologic examination were fixed in 10% neutral buffered formalin, trimmed, and paraffin-embedded, 6 to 7 µm sections cut and stained using hematoxylin and eosin (Luna, 1968).

STATISTICAL ANALYSIS

Comparisons of mean body weights were performed using the Multivariate Analysis of Variance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A one-factorial analysis of variance with multivariate comparisons was used to analyze the clinical chemistry and organ weight data. The histopathology data was analyzed using one of the following nonparametric tests: Fisher's

Exact Test, or if not valid, Yates' Corrected Chi-Square (Zar, 1974). A probability of 0.05 inferred a significant change from controls.

SECTION 3

EXPERIMENTAL RESULTS

PHASE I

Results from the analyses of liver, kidney, fat, muscle, ovary, adrenal, skin, blood, urine, and feces from the dermally treated animals are listed in Table 1. Fyrquel and/or constituents were found in these tissues as early as 1 h following dermal treatment, and relatively high Fyrquel concentrations were noted in all organs by 4 h. Fat, muscle, ovary, and adrenal concentrations peaked at 24 h, then showed a considerable decrease by 48 h. Although Fyrquel concentrations found in the liver and the kidney were comparable, the amount of Fyrquel noted in blood and urine samples collected at 24 h was quite low. Skin concentrations were expectedly high, as it was the site of administration.

TABLE 1. TISSUE CONCENTRATION (μg/g)[a] FOLLOWING DERMAL TREATMENT OF 1.68 g FYRQUEL/kg BODY WEIGHT TO F-344 RATS

Tissue	1 h	4 h	24 h	48 h
Liver	1.62 ± 0.5	11.73 ± 2.4	14.5 ± 5.1	16.20 ± 6.1
Kidney	3.20 ± 1.0	15.41 ± 9.5	8.76 ± 4.2	15.94 ± 7.7
Fat	29.83 ± 13.5	15.41 ± 4.3	70.48 ± 8.1	37.07 ± 4.5
Muscle	43.33 ± 8.1	16.39 ± 3.1	33.92 ± 16.8	21.55 ± 9.2
Ovary	33.18 ± 11.0	32.59 ± 15.2	35.82 ± 6.4	22.93 ± 14.0
Adrenal	20.35 ± 13.3	44.71 ± 10.7	353.57 ± 5.3	8.92 ± 1.2
Skin[b]	8511.72 ± 1554.9	6502.89 ± 1060.6	10203.27 ± 1860.4	6532.27 ± 1741.1
Blood	0.04 ± 0.02	0.17 ± 0.04	0.45 ± 0.07	0.14 ± 0.02
Urine	---	---	3.73 ± 0.76	---
Feces	---	---	271.27 ± 87.96[c]	---

[a] Mean ± SEM, N = 5. Total Fyrquel Concentration.
[b] Dermal Exposure Only.
[c] Diluted Fyrquel.

Results of analyses of the same organs from the gavage-treated animals are listed in Table 2. Liver concentrations achieved were much higher by this route of administration and peaked by 4 h posttreatment. Fat concentrations continued to accumulate and contained a higher concentration of Fyrquel than any organ at 48 h posttreatment. Kidney, ovary, and adrenal concentrations were much lower following oral administration of the hydraulic fluid than concentrations found following dermal exposure.

TABLE 2. TISSUE CONCENTRATION (µg/g)[a] FOLLOWING SINGLE GAVAGE OF 1.68 g FYRQUEL/kg BODY WEIGHT TO F-344 RATS

Tissue	1 h	4 h	24 h	48 h
Liver	22 5 ± 2 5	40 4 ± 3.5	6 5 ± 5.1	0 1 ± <0 1
Kidney	2 3 ± 0 2	7 0 ± 0 5	3 4 ± 0 9	1 6 ± 0 5
Fat	4 9 ± 0 7	20.7 ± 1 8	75 5 ± 9 9	125 0 ± 29 7
Muscle	1 3 ± 0.1	3.7 ± 0 3	25 5 ± 23.2	2 6 ± 0 5
Ovary	7 0 ± 1 3	15 3 ± 1.5	11 6 ± 1 2	15.5 ± 8.5
Adrenal	12 9 ± 1.7	34 2 ± 3.7	18 8 ± 6 2	10 3 ± 1 1
Skin	2 0 ± 0 3	7 7 ± 1 3	14 1 ± 1 6[c]	10.5 ± 2 1
Blood	0 9 ± 0 1	2 1 ± 0 1	0 3 ± 0 1	0 1 ± <0 1
Urine	---	---	0 3 ± <0.1	---
Feces	---	---	51314 0 ± 12036 6[b]	---

[a] Mean ± SEM, N = 5; Total Fyrquel Concentration
[b] Diluted 1:2000
[c] N = 4

Microscopic examination of adrenal, ovary, and thymus tissues following single dermal or oral treatment revealed no treatment-related lesions A few incidental lesions were present but were found in both treated and control animals

PHASE II

No deaths occurred during the 6-week treatment period Fyrquel-treated rats appeared lethargic by Day 7 and remained in that condition throughout the treatment period. A depression in the test-group absolute body weight gains was noted following one week of treatment (Figure 1) The difference in mean body weights of the test animals was statistically different from mean body weights of the control group from Week Two of treatment through Week Six

Daily monitoring of vaginal cytology revealed normal estrous cycles (5-day cycle) for both the control and the Fyrquel-treated animals

Clinical chemistry evaluations performed at necropsy revealed several parameters in the treated rats that were statistically different from control values Total protein and albumin levels were higher than control values ($p<0.01$) following both the 4- and 6-week treatment periods (Table 3) Also, the mean alanine aminotransferase (ALT) value at four weeks was increased ($p<0.01$) over the control value Serum estradiol levels were significantly increased ($p<0.01$) in the treated rats when compared to their respective control group at all treatment intervals (Figure 2)

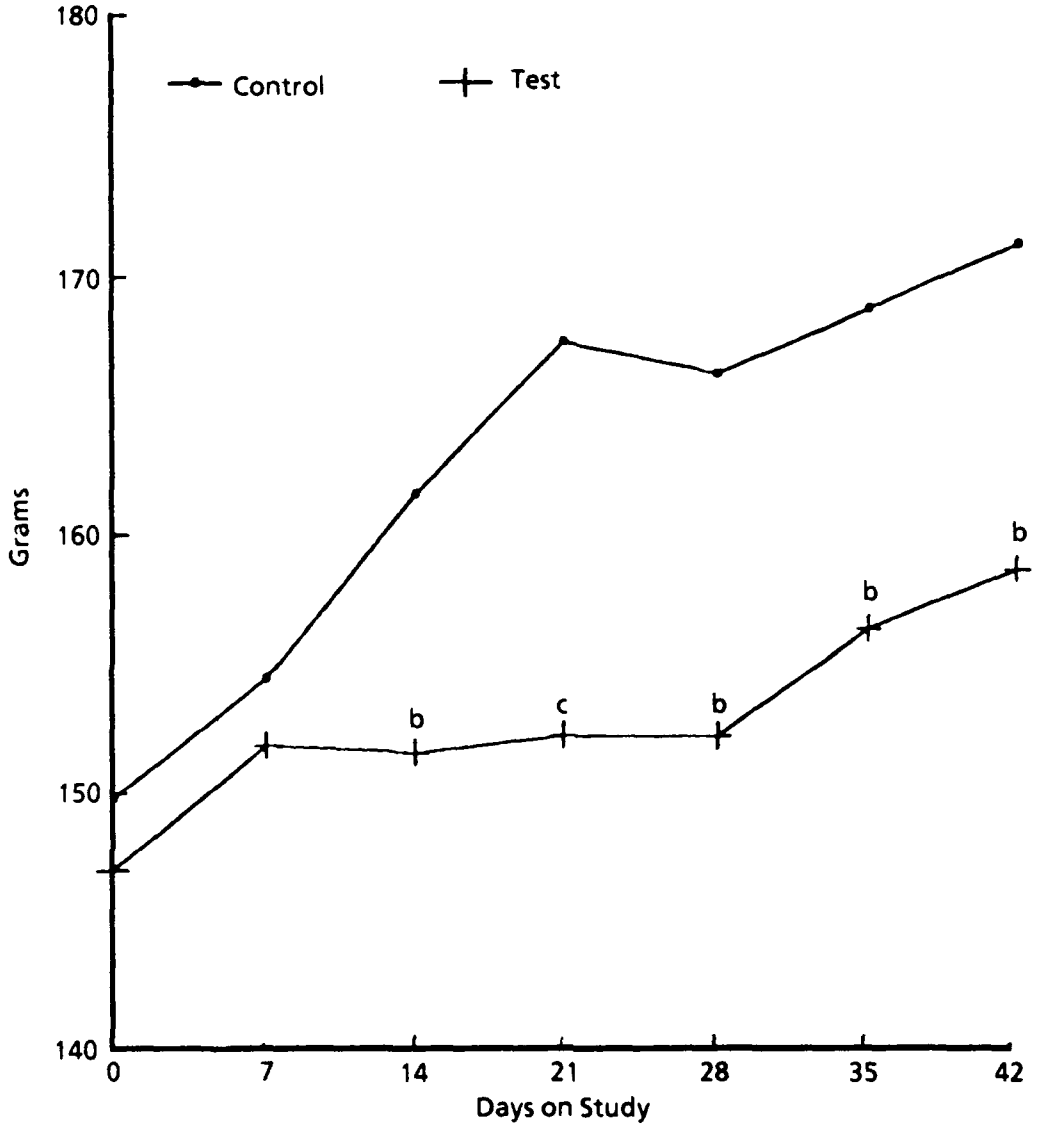

Figure 1. Mean Body Weights for Female F-344 Rats Following Repeated Dermal Administration of Fyrquel Hydraulic Fluid. N = 15 for Days 1 through14; 10 for Days 15 through 28; and 5 for Days 29 through 42.

b = different from control, $p<0.05$.

c = different from control, $p<0.01$.

TABLE 3. MEAN[a] SERUM CHEMISTRY PARAMETERS OF FEMALE F-344 RATS FOLLOWING REPEATED DERMAL TREATMENT WITH FYRQUEL HYDRAULIC FLUID

	2 Weeks		4 Weeks		6 Weeks	
	Control	Test	Control	Test	Control	Test
Total Protein	6.1 ± 0.1	6.4 ± 0.1	5.9 ± 0.1	6.7 ± 0.1[b]	5.1 ± 0.1	6.8 ± 0.1[b]
Albumin	3.7 ± <0.1	4.1 ± 0.1	3.3 ± <0.1	4.0 ± 0.1[b]	3.2 ± 0.1	3.7 ± 0.1[b]
AST	88.4 ± 13.3	74.4 ± 14.4	88.2 ± 3.2	87.6 ± 2.9	88.6 ± 5.6	71.3 ± 5.5[c]
ALT	69.4 ± 3.1	74.8 ± 4.2	58.4 ± 3.8	90.0 ± 3.5[b]	73.0 ± 7.7	81.3 ± 6.1[c]
Globulin	2.4 ± 0.1	2.4 ± <0.1	2.6 ± <0.1	2.7 ± <0.1	2.8 ± 0.1	3.1 ± 0.1

[a] Mean ± SEM, N = 5
[b] Significantly different from control, $p<0.1$, as determined by a one-factorial analysis of variance
[c] N = 4

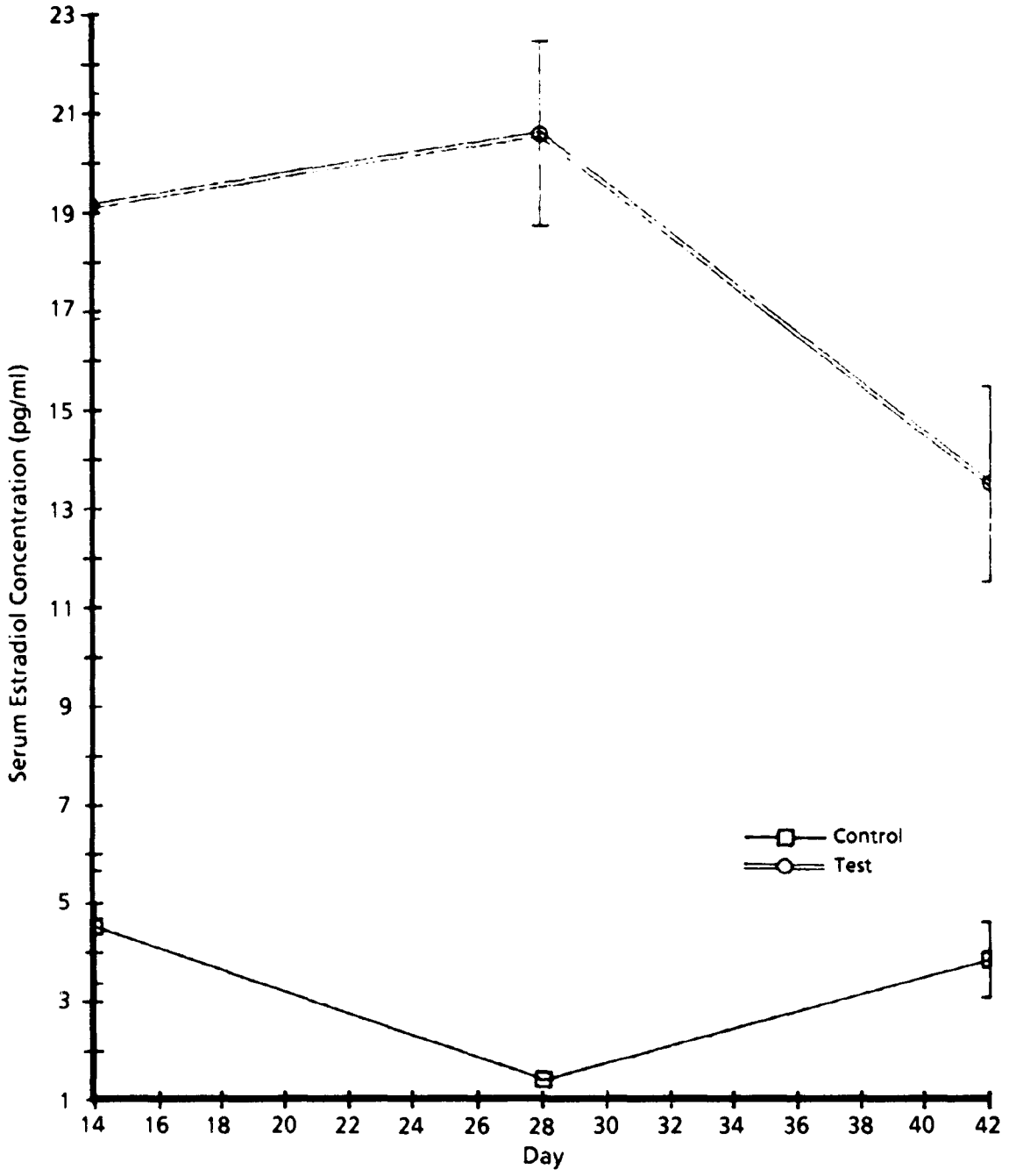

Figure 2. Serum Estradiol Concentrations Following Repeated Dermal Treatment of 1.68 g Fyrquel/kg Body Weight to Female F-344 Rats. Treated levels were significantly higher ($p < 0.01$) than controls at each treatment period.

A significant increase ($p<0.01$) in absolute and relative liver weights occurred by two weeks of treatment and was apparent through the four- and six-week treatment periods (Table 4). Relative liver weights of the H-19457C-treated rats were increased 68 to 77% greater than the relative liver weights of the sesame oil-treated animals. Relative kidney weights were also increased ($p<0.01$) following each treatment period. Relative kidney weights increased by 9, 20, and 17% following the two-, four-, and six-week treatment periods, respectively. The absolute and relative weights of the adrenal glands were also increased throughout the treatment period.

The only gross lesion noted at necropsy was hepatomegaly. This lesion was observed in all treated animals at each necropsy interval.

Hepatocytomegaly was noted in 100% of the treated rats and in none of the control rats (Table 5). This lesion was detected at 14 days, the earliest treatment interval examined. Kidney lesions consisted of foci of proximal convoluted tubules with degeneration and regeneration admixed. Tubular alteration was present in 1/5 rats treated for 28 days and 5/5 treated for 42 days. Tubular alteration was not noted in the rats treated for 14 days or in any of the control animals.

Minimal to mild lipidosis was present in the adrenal glands in approximately equal numbers of control and treated rats. No microscopic lesions were noted in the thymus of any of the study animals. Minimal lipidosis was noted in the ovaries of both treated and control animals.

TABLE 4. ORGAN WEIGHTS[a] (g) AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE RATS FOLLOWING REPEATED DERMAL TREATMENT WITH FYRQUEL HYDRAULIC FLUID

	2 Weeks		4 Weeks		6 Weeks	
	Control	Test	Control	Test	Control	Test
Liver	4.26 ± 0.06	6.93 ± 0.21[c]	4.81 ± 0.13	7.55 ± 0.22[c]	5.35 ± 0.14	8.56 ± 0.40[c]
Ratio[b]	2.99 ± 0.5	5.03 ± 0.06[c]	2.91 ± 0.03	5.16 ± 0.21[c]	3.25 ± 0.05	5.58 ± 0.16[c]
Kidneys	1.10 ± 0.03	1.16 ± 0.05	1.22 ± 0.03	1.30 ± 0.02	1.26 ± 0.45	1.37 ± 0.03
Ratio	0.77 ± 0.01	0.84 ± 0.02[c]	0.74 ± 0.01	0.89 ± 0.01[c]	0.76 ± 0.01	0.89 ± 0.01[c]
Ovaries	0.05 ± <0.01	0.06 ± <0.01	0.06 ± <0.01	0.06 ± <0.01	0.07 ± <0.01	0.07 ± <0.01
Ratio	0.04 ± <0.01	0.04 ± <0.01	0.04 ± <0.01	0.04 ± <0.01	0.04 ± <0.01	0.04 ± <0.01
Thymus	0.21 ± 0.01	0.23 ± 0.01	0.28 ± 0.02	0.20 ± 0.01[c]	0.24 ± 0.01	0.20 ± <0.01[d]
Ratio	0.15 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.13 ± <0.01[d]	0.14 ± 0.01	0.13 ± 0.01
Adrenals	0.05 ± <0.01	0.06 ± <0.01[d]	0.05 ± <0.01	0.06 ± <0.01	0.05 ± <0.01	0.06 ± <0.01[c]
Ratio	0.03 ± <0.01	0.04 ± <0.01[d]	0.03 ± <0.01	0.04 ± <0.01[c]	0.03 ± <0.01	0.04 ± <0.01[c]
Body Weight[e]	143.0 ± 4.3	137.8 ± 4.7	165.2 ± 3.2	146.8 ± 4.5[d]	164.4 ± 1.8	153.3 ± 4.6[d]

[a] Mean ± SEM, N = 5
[b] Organ weight/body weight × 100
[c] Significantly different from control, $p<0.01$, as determined by a one-factorial analysis of variance
[d] Significantly different from control, $p<0.05$, as determined by a one-factorial analysis of variance
[e] Fasted weights

TABLE 5. INCIDENCE (%) OF HISTOPATHOLOGIC FINDINGS FOLLOWING REPEATED DERMAL ADMINISTRATION OF FYRQUEL HYDRAULIC FLUID (N = 5)

	2 Weeks		4 Weeks		6 Weeks	
Tissue/Lesion	Control	Test	Control	Test	Control	Test
Liver: Hepatocytomegaly	0	100	0	100	0	100
Kidneys: Tubular Alteration	0	0	0	20	0	100

SECTION 4

DISCUSSION

No attempt was made to measure the rate or quantitate the amount of hydraulic fluid absorbed through the skin following a single dermal treatment. It was, however, possible to demonstrate the bioavailability of the hydraulic fluid by percutaneous absorption. The fluid was rapidly absorbed to muscle, ovary, and adrenal glands (1 h) and to liver and kidney (4 h), with adrenal and fat concentrations peaking at 24 h. Rates and means of elimination of the hydraulic fluid following the two routes of administration were different. The fluid was more rapidly eliminated following oral administration, but also had more stored in fat after this route of administration.

Organ weights taken at necropsy and histopathologic findings following repeated dermal treatment indicated that liver and kidney, and possibly adrenal gland, are target tissues. Hepatomegaly was present in 100% of the treated rats and correlated precisely with the hepatocytomegaly seen histologically. The enlarged hepatocyte morphology suggests an increased amount of cytoplasmic smooth endoplasmic reticulum and possibly peroxisomes; however, ultrastructural study is required to confirm that increased amounts of these organelles contributed to the cytomegaly. In a previous study (Latendresse, unpublished data 1990), Cytochrome P-450 concentrations have been shown to be significantly increased in rats repeatedly receiving daily oral administration of 1.68 g/kg Fyrquel for 21 days.

The cause of increased kidney weights in treated rats was not apparent microscopically and may be only a consequence of the lower body weights of the treated animals. Consistent histopathology of the adrenal glands was also lacking in the treated animals. Persistent adrenocorticotrophic hormone (ACTH) stimulation of the adrenal cortex could possibly explain the increased adrenal gland weights. Stress caused by a variety of factors, including a toxicant effect, can induce increased corticosteroid production stimulated by ACTH from the pituitary. Microscopic examination of the thymus failed to reveal any explanation for the decrease in thymus weights in treated rats.

The increase in serum estradiol at all treatment intervals was consistent with the results of previous studies (Latendresse, unpublished data 1990). However, the estrous cycles in the treatment group remained normal throughout the study. This same pattern of normal estrous cycles coupled with elevated serum estradiol has been observed in female rats orally dosed with tricresyl phosphate for 21 days (Latendresse, unpublished data 1990). Serum total protein and albumin were also elevated significantly in the treated group. This has been a consistent finding in all triaryl phosphate studies performed in this laboratory. Because albumin is a liver product, increased liver mass in

treated rats may account for the increase in serum albumin. Albumin is the principle estradiol-binding transport protein in the serum. Radioimmunoassay measures total (bound and free) estradiol in the blood. One plausible explanation for elevated estradiol without apparent biological effects on estrous cycles is that the pool of bound estradiol is increased proportionally with the increased serum albumin. Bound estradiol is not biologically active nor can it be excreted. One molecule of albumin can carry several molecules of estradiol. Therefore, a small increase in albumin could result in a much larger increase in the bound estradiol pool.

Studies have shown that rat skin, in general, is more permeable to topically applied compounds than human skin (Bartek et al., 1972). Therefore, percutaneous absorption in the rat is not necessarily predictive of that in humans. However, this study does demonstrate the possibility of systemic toxic effects following percutaneous exposure to this hydraulic fluid and that the target organs are the same as those noted following the long-term oral study which caused adverse reproductive effects.

SECTION 5

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APPENDIX A

GC/MS ANALYSIS OF FYRQUEL

MATERIALS AND METHODS

Fyrquel 220® (Mil-H-19457C, Lot 3820J-1, CAS # 115-86-6), provided by the U.S. Navy, was manufactured by AKZO Chemicals, Inc (Chicago, IL). Hexane was obtained from Fisher Scientific (Fisher H303-4, Fairlawn, NJ), ethyl acetate from Burdick & Jackson (B&J 100-4, Muskegon, MI), and sodium chloride from Aldrich Chemical Co. (Aldrich 7647-14-5, Milwaukee, WI). The internal standard, tri-*m*-tolyl phosphate, was obtained from Eastman Kodak Co. (Kodak P2758, Rochester, NY). The chromatography column used was a 12-m x 0.2-mm HP-1 column supplied by Hewlett-Packard Co. (Avondale, PA).

Mass spectra were obtained using a Hewlett-Packard 5890 GC with a Hewlett-Packard 7673A Autosampler. The GC was interfaced to a Hewlett-Packard 5970 MSD quadrupole mass spectrometer equipped with a 70 eV electron impact source. The electron optics were calibrated using perfluorotri-*n*-butyl amine and mass spectra were obtained scanning from 50-700 amu. The chromatographic data was processed using a Hewlett-Packard 59970 Chem Station using version 3.1 of the MSD software. A Tekmar Tissumizer was used for the homogenization of tissue samples (Tekmar Co., Cincinnati, OH), and a Haake-Buchler vortex-evaporator (Haake-Buchler, Inc., Saddlebrook, NJ) was used for the extraction of biological samples. Plastic scintillation vials were obtained from Baxter Scientific Products (Baxter R 2555-3, Obetz, OH) and 4 mL glass vials were obtained from Kimble Glass (Kimble 60810 G, Toledo, OH). Ethylenediaminetetraacetic acid (EDTA) tubes were obtained from Becton Dickenson (47 mm x 10.25 mm, Rutherford, NJ).

PROCEDURE

Samples of liver, kidney, fat, muscle, skin, and feces, approximately 0.5 g in weight, were placed in 20 mL scintillation vials and homogenized in 5 mL of 0.9% saline for approximately 5 min. Urine samples were processed in this manner without homogenizing. Similarly, 0.1-g samples of adrenal and ovary were homogenized in 1 mL of 0.9% saline using 4-mL glass vials. Blood samples were initially collected in EDTA tubes. A 1-mL sample of whole blood was transferred to a 4-mL glass vial for extraction.

All samples were extracted with a 1:1 (v/v) mixture of hexane-ethyl acetate. Equal volumes of hexane-ethyl acetate were added to each vial. The internal standard, added to the hexane-ethyl acetate layer, was tri-*m*-tolyl phosphate (20 µg/mL). Samples extracted with 1 mL of solvent received

20 µL of internal standard, and samples extracted with 5 mL of solvent received 100 µL of internal standard. The vials were then vortexed overnight at room temperature at moderate speed.

After vortexing, the samples were centrifuged at 2000 x g for 20 min. The organic layer was removed and transferred to an autosampler vial for analysis by GC/MS with selected-ion-monitoring. The chromatography conditions used for the analysis are shown in Table A.

TABLE A. CHROMATOGRAPHY CONDITIONS FOR GC/MS ANALYSIS

Injector:	290 °C
Detector:	300 °C
Initial Col Temp:	200 °C
Initial Time:	15 sec
Rate:	10 °C/min
Final Col Temp:	290 °C
Hold:	15 min
Transfer Line:	280 °C
Carrier Gas:	Helium

During the first 15 sec of the chromatography analysis the splitless valve was on. This allowed samples to concentrate at the head of the column prior to analysis. The parameters used for selected-ion monitoring are shown in Table B.

TABLE B. INSTRUMENT PARAMETERS FOR SELECTED-ION-MONITORING

Ion m/z	Dwell time (msec)
326	100
367	100
368	50
423	100

The base peak of triphenyl phosphate was monitored at 326 m/z. The ions at 367 and 423 m/z monitored the base peaks of *p*-*t*-butylphenyl diphenyl phosphate and bis-(*p*-*t*-butylphenyl) phenyl phosphate, respectively. The base peak of the internal standard, tri-*m*-tolyl phosphate, was monitored at 368 m/z.

RESULTS

The total-ion-chromatogram obtained from the GC/MS analysis of a liver extract containing Fyrquel is shown in Figure A. The retention times of the major components of Fyrquel are shown in Table C.

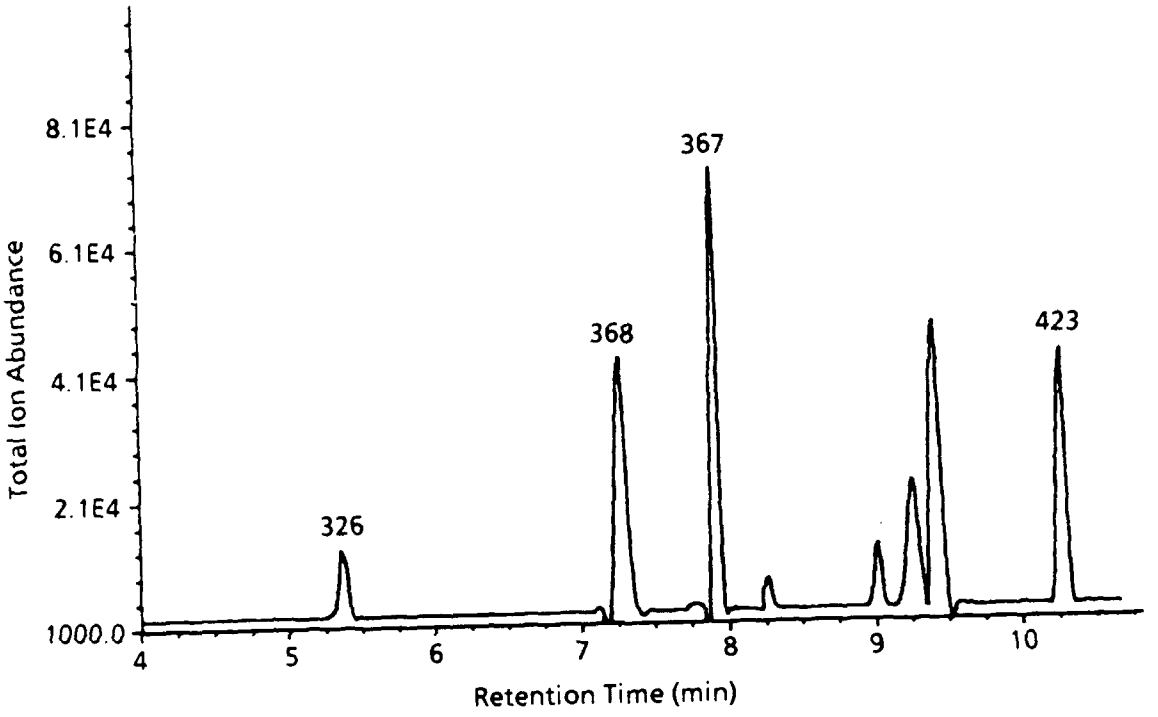

Figure A. Total Ion Chromatogram of Fyrquel Components from a Liver Extract: triphenyl phosphate (5.37 min, ion 326 m/z), tri-*m*-tolyl phosphate (internal standard, 7.28 min, ion 386 m/z), *p*-*t*-butylphenyl diphenyl phosphate (7.92 min, ion 367 m/z) and bis (*p*-*t*-butylphenyl) phenyl phosphate (10.26 min, ion 423 m/z).

TABLE C. RETENTION TIMES OF MAJOR FYRQUEL COMPONENTS

Component	Ret t (min)	Wt. %
Triphenyl phosphate	5.37	13.2
p-*t*-Butylphenyl diphenyl phosphate	7.92	52.6
Bis(*p*-*t*-butylphenyl) phenyl phosphate	10.26	30.2

These three components account for 96% (by weight) of all of the components of Fyrquel (AKZO, 1989). Their mass spectra are shown in Figures B through D. The first component, triphenyl phosphate has a base peak at 326 m/z, as shown in Figure B. The second component, *p-t*-butylphenyl diphenyl phosphate, has a base peak at 367 m/z, as shown in Figure C and the third component, bis(*p-t*-butylphenyl) phenyl phosphate, has a base peak at m/z 423, as shown in Figure D. The finding that the major components of Fyrquel have base peak ions at high m/z values facilitates the use of selected-ion-monitoring.

However, there were complications when applying the GC/MS method with selected-ion-monitoring to biological samples. Specifically, there was a matrix effect that gave an attenuated instrument response when analyzing extracts from biological samples. Biological samples were observed to have anomalously high peak areas for their respective concentrations when compared to standards in hexane-ethyl acetate solvent. Therefore, it is not possible to use peak areas from a standard curve in hexane-ethyl acetate solvent to directly quantitate peak area responses from biological samples.

An internal standard was used to correct for this matrix effect. The internal standard used was tri-*m*-tolyl phosphate (Figure E). This compound had a retention time of 7.28 min and a base peak at 368 m/z. Peak area ratios were used to quantitate biological samples. Standard curves were prepared from homogenates of the same tissue which were analyzed. The on-column limits of detection of the Fyrquel components were: 7 pg for triphenyl phosphate, 26 pg for *p-t*-butylphenyl diphenyl phosphate, and 15 pg for bis(*p-t*-butylphenyl) phenyl phosphate.

RECOVERY DATA

Recovery experiments were performed to validate the method. Tissue homogenates were prepared in 0.9% saline using 0.1 g of tissue per 1 mL of saline. Blood and urine samples were spiked directly with Fyrquel. The samples were spiked to give concentrations ranging from 50 ng/mL to 10,000 ng/mL total Fyrquel. The extraction solvent, hexane-ethyl acetate, was added followed by the addition of the internal standard. The extracted Fyrquel samples were compared to control matrix standards made up to the same concentrations in a control matrix extract. The control matrix extract was composed of the extracted homogenate of the same type of tissue. The recovery data are shown in Table D. A level of 50 to 100 ng/mL (GC/MS analyzed extract) corresponds to a tissue level of 0.5 to 1.0 µg/g. Very few samples fall into this range, therefore, the low extraction efficiency does not effect the majority of the samples.

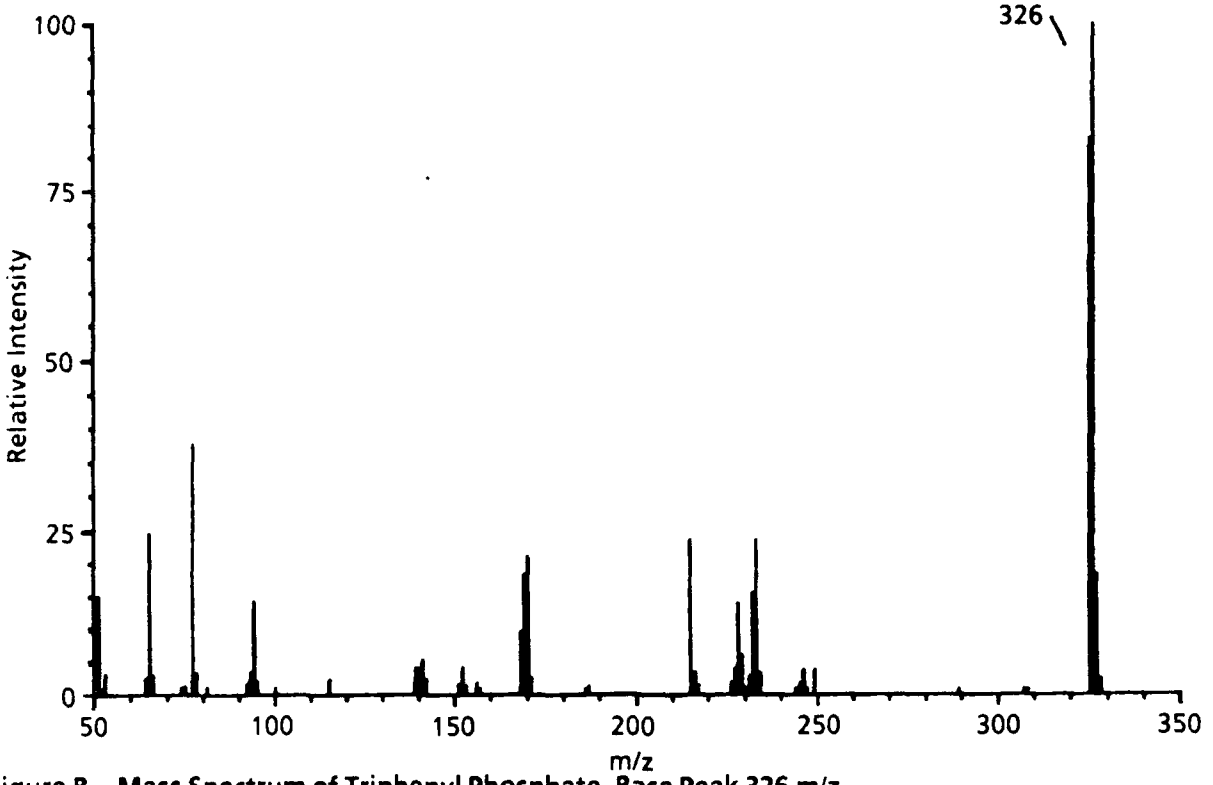

Figure B. Mass Spectrum of Triphenyl Phosphate, Base Peak 326 m/z.

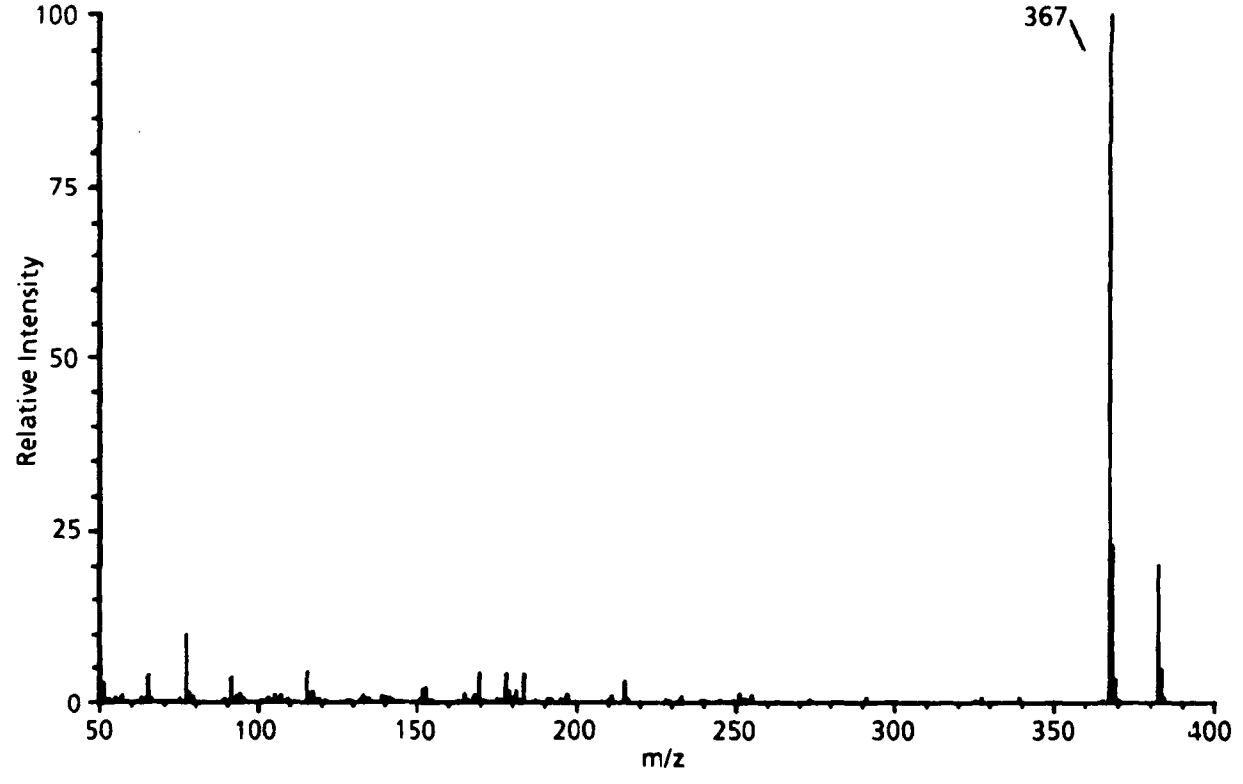

Figure C. Mass Spectrum of *p*-t-Butylphenyl Phosphate, Base Peak 367 m/z.

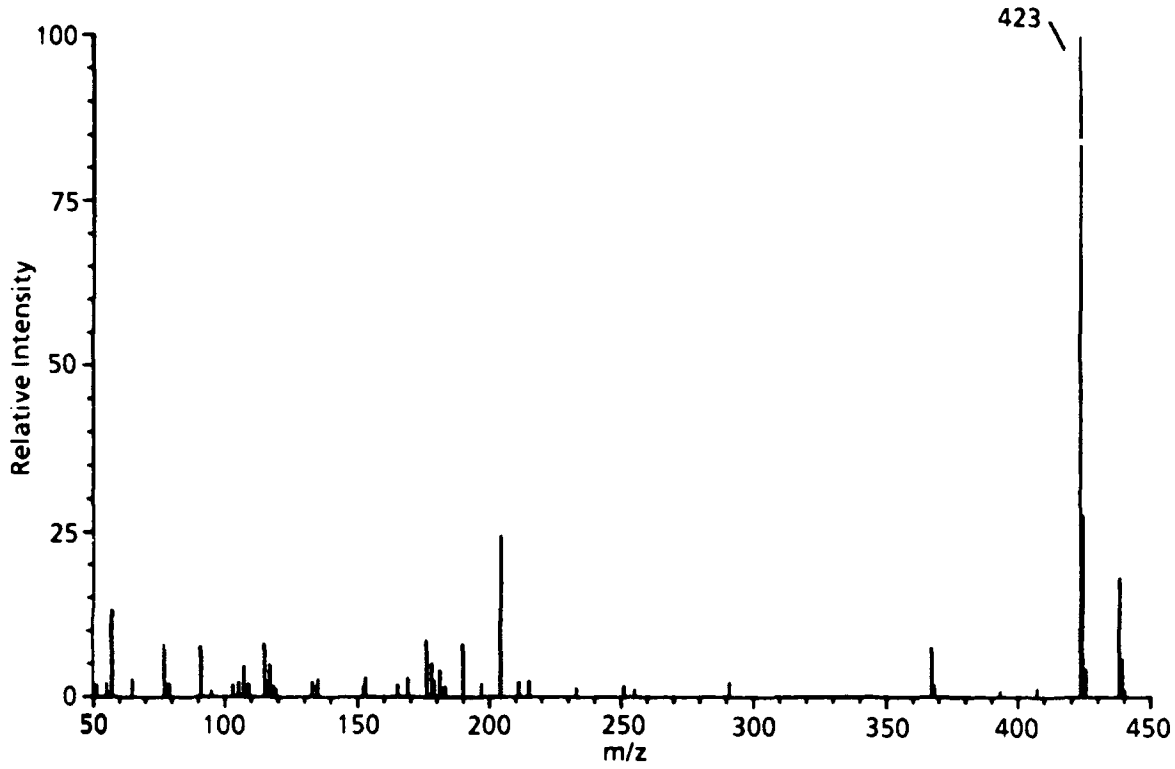

Figure D. Mass Spectrum of Bis (*p*-t-butylphenyl) Phenyl Phosphate, Base Peak 423 m/z.

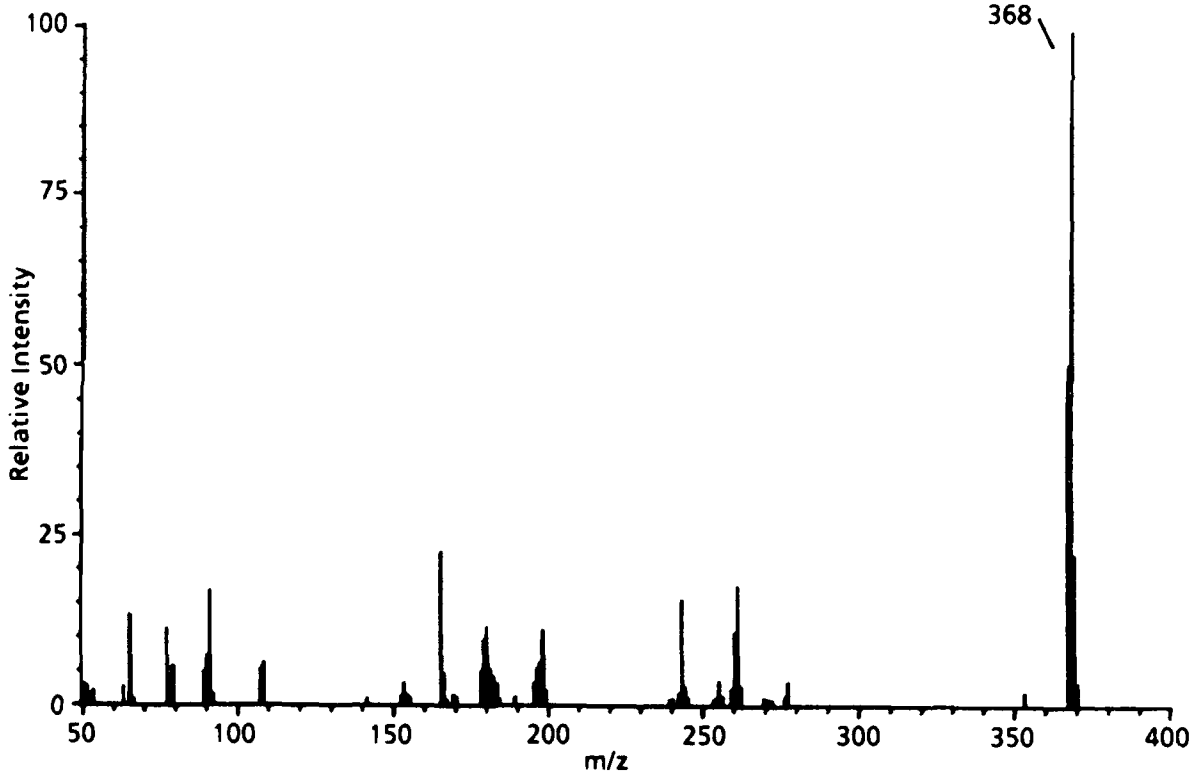

Figure E. Mass Spectrum of Internal Standard, Tri-*m*-tolyl Phosphate, Base Peak 368 m/z.

TABLE D. FYRQUEL RECOVERY DATA

Sample	ng/mL (Total Fyrquel)	% Recovery
Liver	50	21
	100	42
	1000	98
	10,000	102
Kidney	50	113
	100	96
	1000	98
	10,000	98
Fat	50	118
	100	95
	1000	100
	10,000	98
Muscle	50	107
	100	106
	1000	105
	10,000	106
Skin	50	148
	100	115
	1000	92
	10,000	89
Ovary	500	103
Adrenal	500	105
	1000	103
Blood	50	102
	100	109
	1000	91
	10,000	93
Feces	50	59
	100	102
	1000	101
	10,000	96
Urine	50	0
	100	42
	1000	101
	10,000	80

REPEATABILITY DATA

The repeatability of replicate determinations was measured at two levels of total Fyrquel. Tissue homogenates were spiked with Fyrquel at levels of 500 and 1000 ng/mL (total Fyrquel). The various types of biological matrices were analyzed, with the exceptions of ovary and adrenal. Four duplicate samples were used to measure the repeatability. The results are shown in Table E.

TABLE E. FYRQUEL REPEATABILITY DATA

Sample	ng/mL (Total Fyrquel)	Repeatability[a] ng/mL (N = 4)
Liver	500	493 ± 27 8
	1000	1047 ± 13 6
Kidney	500	491 ± 2 6
	1000	995 ± 27.4
Fat	500	499 ± 9.7
	1000	957 ± 29.2
Muscle	500	505 ± 14.2
	1000	1010 ± 23 4
Skin	500	481 ± 15 3
	1000	903 ± 25 5
Blood	500	529 ± 18 1
	1000	1019 ± 62 8
Feces	500	488 ± 24 1
	1000	998 ± 33 4
Urine	500	467 ± 9 5
	1000	953 ± 42 2

a [illegible] ± Standard Deviation

MATRIX EFFECT

A matrix effect was observed when analyzing Fyrquel components via GC/MS This effect produced an attenuated response in the presence of the hexane-ethyl acetate extractable components of a biological sample The GC/MS analysis of this study included ten different types of biological samples An investigation was undertaken to establish whether the matrix effect is similar in all tissues or whether different tissues have different effects

To achieve this the standard curves for all ten tissues were analyzed in duplicate by GC/MS The standard curves for the individual components were analyzed by a general model to determine whether the curve was linear, quadratic, or a higher order polynomial The general model was used to compute an overall variance for each of the three Fyrquel components The coefficients from each standard curve of a Fyrquel component in different tissues were tested against each other This was done pair-wise with Bonferroni's correction for multiple comparisons (Miller, 1966) Statistical significance was accepted at $p \leq 0.05$ The results of the analysis, summarized in the Tables F through H, indicate that the matrix effect is significantly different for all ten of the different tissues Based on these statistics, it is necessary to run standards in their respective matrix in order to obtain an accurate

analysis. Inspection of Tables F through H reveals that not all components of all tissues are significantly different. However, there is not a single pair of tissues which is not significantly different for all three Fyrquel components.

TABLE F. TABLE OF BONFERRONI COMPARISON OF TISSUE SLOPES FOR FYRQUEL COMPONENT 326 m/z TRIPHENYL PHOSPHATE

	Kidney	Liver	Muscle	Adrenal	Ovary	Blood	Skin	Feces	Urine	Fat
Kidney	–	0.05	NS	0.01	0.05	0.01	0.01	0.01	0.01	0.01
Liver		–	0.01	0.01	0.01	0.01	0.05	0.01	0.01	0.01
Muscle			–	0.01	0.05	0.01	0.01	0.01	0.01	0.01
Adrenal				–	0.01	0.01	0.01	0.01	0.01	0.01
Ovary					–	0.01	0.01	0.01	0.05	0.01
Blood						–	0.01	0.01	0.01	0.01
Skin							–	0.01	0.01	0.01
Feces								–	0.01	0.01
Urine									–	0.05
Fat										–

NS = Comparison not significant

TABLE G. TABLE OF BONFERRONI COMPARISON OF TISSUE SLOPES FOR FYRQUEL COMPONENT 367 m/z *p*-*t*-BUTYLPHENYL DIPHENYL PHOSPHATE

	Kidney	Liver	Muscle	Adrenal	Ovary	Blood	Skin	Feces	Urine	Fat
Kidney	–	NS	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Liver		–	0.01	0.01	0.01	0.01	0.01	0.01	NS	0.01
Muscle			–	0.01	0.01	0.01	0.01	0.01	0.01	NS
Adrenal				–	0.01	0.01	0.01	0.01	0.01	0.01
Ovary						0.01	0.01	0.01	0.01	0.01
Blood						–	0.01	0.01	0.01	0.01
Skin							–	NS	0.01	NS
Feces								–	0.01	0.01
Urine									–	0.01
Fat										–

NS = Comparison not significant

TABLE H. TABLE OF BONFERRONI COMPARISON OF TISSUE SLOPES FOR FYRQUEL COMPONENT 423 m/z Bis(*p*-*t*-BUTYLPHENYL) PHENYL PHOSPHATE

	Kidney	Liver	Muscle	Adrenal	Ovary	Blood	Skin	Feces	Urine	Fat
Kidney	–	NS	0 01	0 01	0 01	0 01	0 01	0 01	0 01	0 01
Liver		–	0 01	0 01	0 01	0 01	0 05	0 01	0 01	0 01
Muscle			–	0 01	0 01	0 01	0 01	0 01	0 01	0 01
Adrenal				–	0 01	0 01	0 01	0 01	0 01	0 01
Ovary						0 01	0 01	0 01	0 01	0 01
Blood						–	0 01	NS	0 01	0 01
Skin							–	0 01	0 01	0 01
Feces								–	0 01	0 01
Urine									–	0 01
Fat										–

[illegible] comparison not significant

DERMAL AND ORAL EXPOSURE

The combined GC/MS method with selected-ion-monitoring was used to determine tissue levels of Fyrquel from exposed rats. The results of the dermal and oral exposure studies are summarized in Tables 2 and 3 of the technical report. Study samples which appeared to have unusually high values were confirmed by obtaining complete mass spectra. Complete mass spectra were obtained scanning from 50-700 amu. The samples were:

Dermal Dose	Muscle	24 h	#16
Dermal Dose	Adrenal	24 h	#16
Oral Dose	Muscle	24 h	#52
Oral Dose	Ovary	48 h	#35
Oral Dose	Skin	24 h	#52

Analysis of these samples showed good agreement between the mass spectra of authentic standards and the mass spectra obtained from the study samples. This indicates that the values are accurate and cannot be attributed to coeluting impurities.

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QUALITY ASSURANCE

The study, "Dermal Absorption of H-19457C Hydraulic Fluid," was conducted by the ManTech Environmental Technology, Inc., Toxic Hazards Research Unit under the guidance of the Environmental Protection Agency's Good Laboratory Practices Guidelines, 40CFR PART 792. No claim will be made that this was a "GLP" study as no attempt was made to adhere to the strict requirements of these guidelines. The various phases of this study were inspected by members of the Quality Assurance Unit. Results of these inspections were reported directly to the Study Director at the close of each inspection.

DATE OF INSPECTION:	**ITEM INSPECTED:**
August 17, 1990	Animal identification procedure
August 28, 1990	Single dermal dosing Sacrifice, 1h post-dose Sacrifice, 4h post-dose
September 18, 1990	Repeated dermal: weighing, dose #1 Repeated dermal: dose removal
October 2, 1990	Repeated dermal: weighing, dose #15
October 30, 1990	Repeated dermal: weighing, 42-day sacrifice

The Quality Assurance Unit has determined by review process that this report accurately describes those methods and standard operating procedures required by the protocol and that the reported results accurately reflect the raw data obtained during the course of the study. No discrepancies were found that would alter the interpretation presented in this Final Report.

M. G. Schneider
QA Coordinator
Toxic Hazards Research Unit

Date 29 May 91