C-271

# DETERMINATION OF TIME TO STEADY-STATE LEVEL IN BLOOD DURING INHALATION EXPOSURE OF BENZENE BY RATS AND MICE

## PART I

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## SUMMARY

The levels of benzene in rat and mouse blood, and selected metabolites phenol, catechol, and hydroquinone, were determined at exposure intervals of 0, 1, 2, 4, 8 and 12 hours at a 300 ppm exposure level. The mean levels of benzene in rat blood were < 1.0, 4.7, 4.7, 5.7, 5.3 and 7.0 ppm at intervals of 0, 1, 2, 4, 8 and 12 hours, respectively. No levels of free phenol, catechol and hydroquinone at or above the limit of detection (1.0, 0.5 and 0.1 ppm, respectively) were determined in rat blood at any of the exposure intervals. The mean levels of benzene in mouse blood were < 1.0, 3.7, 3.0, 2.4, 3.0 and 1.3 ppm at intervals of 0, 1, 2, 4, 8 and 12 hours, respectively. The mean levels of free phenol in mouse blood were 2.0, 2.4, 2.1, 2.3, 2.5 and 2.3 ppm at intervals of 0, 1, 2, 4, 8 and 12 hours, respectively. No levels of free catechol and hydroquinone at or above the limit of detection (0.5 ppm) were determined in mouse blood at any of the exposure intervals.

The levels of conjugated phenol, catechol and hydroquinone were also determined in rat and mouse blood previously extracted for the free compounds. The mean levels of conjugated phenol in rat blood were < 1.0, 3.0, 5.3, 4.2, 7.1 and 4.7 ppm at intervals of 0, 1, 2, 4, 8 and 12 hours, respectively. No levels of conjugated catechol and hydroquinone at or above the limit of detection (0.5 ppm) were determined in rat blood at any of the exposure intervals. The mean levels of conjugated phenol in mouse blood were 2.7, 7.2, 8.7, 8.4, 9.1 and 3.7 ppm at intervals of 0, 1, 2, 4, 8 and 12 hours, respectively. No levels of conjugated catechol and hydroquinone at or above the limit of detection (0.5 ppm) were determined in mouse blood at any of the exposure intervals. The mean levels of conjugated phenol in mouse blood were greater than the benzene levels in mouse blood.

A group of four rats and sixteen mice was sham-exposed to air only for 12 hours in a separate room from the test animals and bled at termination of the exposure. No detectable levels of benzene, phenol, catechol and hydroquinone were observed in the rat blood. However, levels of 2.5, 2.5, 2.3 and 2.4 ppm of free phenol were detected in the mouse blood. These residues are probably related to naturally occurring phenol in the blood. No detectable levels of benzene, catechol and hydroquinone were observed in the mouse blood. No detectable levels of conjugated

phenol, catechol and hydroquinone were observed in the rat and mouse blood of these control animals with the exception of three apparent artifacts.

These data indicate a time to steady-state level of benzene in rat and mouse blood of approximately one hour. Animals for Phase II of this study will be exposed to varying concentrations of benzene for 6 hours.

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## 1.0 INTRODUCTION

An inhalation exposure study of benzene in rats and mice was performed by the Inhalation Toxicology Department of Bio/dynamics Inc. (details are given in Part I of this report). Rats and mice were exposed to a constant concentration of approximately 300 ppm for a period of 12 hours. At intervals of 0, 1, 2, 4, 8 and 12 hours, blood was drawn from the animals via the retro-orbital sinus. The blood samples were then analyzed for benzene and selected metabolites, phenol, catechol and hydroquinone and their conjugates, to determine the exposure time necessary to reach steady-state blood levels.

## 2.0 EXPERIMENTAL

### 2.1 Materials

Benzene, supplied by the Chemical Manufacturers Association, was used for this study. The results of a purity analysis to confirm the suitability of this benzene for inhalation studies are given in Table I. Methylene chloride (Baker, Resi-analyzed) was re-distilled in glass for use in benzene extraction from blood. All other solvents were pesticide-grade or equivalent.

### 2.2 Test Material Delivery

The benzene was placed into a 1000 milliliter erlenmeyer filtering flask and pumped into a three-neck, round-bottom flask using an FMI fluid metering pump (Model RPG-20). The conducting tubing was virtually all glass to avoid possible contamination of the benzene by plasticizers. The round-bottom

flask was heated to 40°C for the mice exposure and 34°C for the rat exposure with a heating mantle and variable transformer, to ensure complete volatilization of the benzene. The vapor-laden air was drawn into the horizontal intake of a one-cubic meter stainless steel and glass exposure chamber, where it was diluted with room air to reach the desired exposure concentration. The exposures lasted for 12 hours.

The chamber atmosphere was continuously monitored throughout the entire exposure, with a Miran IA Ambient Air Infrared Analyzer. Absorbance readings were recorded and compared to a calibration curve generated using the same instrument and instrumental settings. This comparison of measured absorbances to the calibration curve yielded the chamber atmosphere concentration.

The stainless steel and glass exposure chambers, which housed the test rats and mice, were operated dynamically at air flow rates of 219 and 205 liters per minute, respectively, and had a total volume of one cubic meter. This provided for one complete air change every 4.6 and 4.9 minutes, and 99% equilibration times of 21.0 and 22.5 minutes, respectively.

2.3 Test Animals

The test animals consisted of 20 male, Sprague-Dawley, rats obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts (238 g to 265 g) and 80 male, SCH:ARS ($ICR_f$) Swiss Albino, mice obtained from ARS-Sprague-Dawley,

Madison, Wisconsin (24 g to 36 g). Information regarding animal housing, etc., is given in Table XIV. Rats and mice were exposed on separate days (rats on 4/26/79 and mice on 6/12/79). The test animals were removed from exposure through a vented air lock after 1, 2, 4, 8 and 12 hours of exposure, eye bled and sacrificed (ethyl ether). Mice were exposed in four groups of four, for removal at each bleeding interval, while four rats were exposed individually for removal at each interval. This was to ensure sufficiently large, pooled blood samples from the mice for the analytical procedures. The times they were removed from the chamber, times of orbital bleeding, times of spiking the blood samples with internal standards and pre/post-exposure physical observations were recorded. The blood samples, collected under light ether anesthesia, were collected in heparinized, glass tubes, shaken for one minute, placed in ice and subsequently analyzed, as described in Part II.

2.4 Control Animals

In order to provide control data for this experiment, two similar groups of four rats (237 g to 263 g) or sixteen mice (25 g to 32 g) were included in the experimental design. One control group was bled and sacrificed immediately pre-exposure. The other control group was sham-exposed to air only for 12 hours in a separate room from the test animals and bled at the termination of exposure. All other handling of the control animals was the same as that for the test animals.

## 2.5 Preliminary Methodology Investigations

### 2.5.1 Free Benzene and Phenol

Recoveries of $^{14}$C-benzene and $^{14}$C-phenol (Ca. 11,000 cpm) fortified into whole blood were used to ascertain the appropriate solvent systems for quantitative extraction. Dichloromethane extracted $^{14}$C-benzene from whole blood in a one-pass extraction with 2 ml of blood and 2 ml of solvent. Recoveries of $^{14}$C-activity ranged from 63 - 90%. Diethyl ether extracted $^{14}$C-phenol from whole blood after 3 passes with equal volumes of blood to solvent. Recoveries ranged from 85 - 91% (see Table II). The C.I.I.T. method employing methanol gave lower recoveries in our hands.

It should be noted that this method will extract free but not conjugated phenols. Acid hydrolysis of whole blood to liberate conjugated phenols resulted in co-extractants which could not be separated by gas chromatography.

The question of whether benzene might be lost by volatilization during the eye bleeding was addressed by fortifying fresh whole rat blood at 37°C with $^{14}$C-benzene and transferring the blood, dropwise, to a vacutainer at 0°C. Dichloromethane extraction of this sample resulted in recoveries of $^{14}$C-activity of 74, 75 and 76%.

### 2.5.2 Conjugated Phenols

Since both sulfate and glucuronide conjugates of phenol are known to be present in urine, it was assumed that

a method for the determination of both phenolic conjugates in blood would be needed. Furthermore, it had been shown that these conjugates in urine are not easily hydrolyzed by acids. Heating whole blood cells with an equivalent volume of 6N $H_2SO_4$ for 1 hour in a boiling water bath resulted in extracts which contained large amounts of co-extractants on gas chromatographic anlaysis. Thus, an enzymatic approach to deconjugation was employed. As model compounds, we chose $^3$H-estradiol glucuronide and $^3$H-estrone sulfate. The results of incubations of whole blood fortified with these conjugates, followed by solvent extraction are summarized in Table III. Experiment No. 3 was found to give the best deconjugation conditions and, thus, these conditions were employed for this study.

2.6 Determination of Benzene in Rat Blood by Gas Chromatographic Analysis

2.6.1 Sample Preparation

Immediately upon bleeding from the rat, heparinized rat blood was fortified with fluorobenzene at 5 ug/ml of blood. A 2 ml aliquot of the internal standardized blood was extracted with 1 ml of dichloromethane, and the mixture was centrifuged at 4000 r.p.m. for 10 minutes. The dichloromethane (lower) phase was transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

2.6.2 Gas Chromatographic Analysis

A standard curve was generated by injecting known amounts of benzene (1.0 - 40.0 ng) in combination with a constant amount of fluorobenzene (10.0 ng). Standards were plotted as the peak height ratio of benzene to fluorobenzene vs. ng of benzene injected. Residues were determined by comparing peak height ratios of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 1 - 3, respectively. Gas chromatographic conditions are given in Table IV. The limit of detection is 1.0 ppm.

2.7 Determination of Benzene in Mouse Blood by Gas Chromatographic Analysis

2.7.1 Sample Preparation

Immediately upon bleeding from the mouse, heparinized mouse blood was fortified with fluorobenzene at 5 ug/ml of blood. A 1 ml aliquot of the internal standardized blood was extracted with 0.5 ml of dichloromethane, and the mixture was centrifuged at 4000 r.p.m. for 10 minutes. The dichloromethane (lower) phase was transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

2.7.2 Gas Chromatographic Analysis

A standard curve was generated by injecting known amounts of benzene (1.0 - 40.0 ng) in combination with

a constant amount of fluorobenzene (10.0 ng). Standards were plotted as the peak height ratio of benzene to fluorobenzene vs. ng of benzene injected. Residues were determined by comparing peak height ratios of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 1, 2 and 4, respectively. Gas chromatographic conditions are given in Table IV. The limit of detection is 1.0 ppm.

2.8 Determination of Phenol, Catechol and Hydroquinone in Rat Blood by Gas Chromatographic Analysis

2.8.1 Sample Preparation - Phenol

Immediately upon bleeding from the rat, heparinized rat blood was fortified with p-cresol at 7.5 ug/ml of blood. A 1 ml aliquot of the internal standardized blood was extracted three times with 1 ml portions of ethyl acetate. Each extraction was centrifuged at 4000 r.p.m. for 10 minutes, and the ethyl acetate (upper) phases were combined. The combined extract was divided into a 2 ml aliquot for phenol analysis and a 1 ml aliquot for combined catechol and hydroquinone analysis.

For phenol analysis, the 2 ml aliquot of blood extract was evaporated to 0.5 ml under dry nitrogen and transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

2.8.2 Gas Chromatographic Analysis - Phenol

A standard curve was generated by injecting known amounts of phenol (1.0 - 40.0 ng) in combination with a constant amount of p-cresol (10.0 ng). Standards were plotted as the peak height ratio of phenol to p-cresol vs. ng of phenol injected. Residues of phenol were determined by comparing peak height ratios of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 5 - 7, respectively. Gas chromatographic conditions are given in Table V. The limit of detection is 1.0 ppm.

2.8.3 Sample Preparation - Catechol and Hydroquinone

For catechol and hydroquinone analysis, the 1 ml aliquot of blood extract was evaporated almost to dryness with dry nitrogen. The residue was caused to react with 100 ul of trifluoroacetic anhydride (TFAA) for 30 minutes at 50°C. One ml of benzene and 4.0 mls of pH 6 phosphate buffer were added to the derivative solution, the solution was mixed on a Vortex mixer for 30 seconds and centrifuged for 5 minutes at 4000 r.p.m. An aliquot of benzene (upper) phase was injected into a Tracor 222 gas chromatograph equipped with a $Ni^{63}$ electron capture detection system.

2.8.4 Gas Chromatographic Analysis - Catechol and Hydroquinone

A standard curve was generated by injecting known amounts of combined catechol and hydroquinone (0.050 - 0.50 ng). Standards were plotted as peak height vs. ng injected. Residues

of catechol and hydroquinone were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 8 - 10, respectively. Gas chromatographic conditions are given in Table VI. The limit of detection is 0.5 ppm for catechol and 0.1 ppm for hydroquinone.

2.9 Determination of Phenol, Catechol and Hydroquinone in Mouse Blood by Gas Chromatographic Analysis

Immediately upon bleeding from the mouse, heparinized mouse blood was fortified with p-cresol at 7.5 ug/ml of blood. A 0.5 ml aliquot of the internal standardized blood was extracted three times with 1 ml portions of ethyl acetate. Each extraction was centrifuged at 4000 r.p.m. for 10 minutes, and the ethyl acetate (upper) phases were combined. The combined extract was divided into a 2 ml aliquot for phenol analysis and a 1 ml aliquot for combined catechol and hydroquinone analysis.

For phenol analysis, the 2 ml aliquot of blood extract was evaporated to 0.25 ml under dry nitrogen and transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

2.9.2 Gas Chromatographic Analysis - Phenol

A standard curve was generated by injecting known amounts of phenol (1.0 - 40.0 ng) in combination with a constant amount of p-cresol (10.0 ng). Standards were plotted as the peak height ratio of phenol to p-cresol vs. ng of phenol injected. Residues of phenol were determined by comparing peak

height ratios of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures, 5, 6 and 11, respectively. Gas chromatographic conditions are given in Table V. The limit of detection is 1.0 ppm.

2.9.3 Sample Preparation - Catechol and Hydroquinone

For catechol and hydroquinone analysis, the 1 ml aliquot of blood extract was evaporated almost to dryness with dry nitrogen. The residue was caused to react with 100 ul of TFAA for 30 minutes at 50°C. One-half ml of benzene and 4.0 mls of pH 6 phosphate buffer were added to the derivative solution, the solution was mixed on a Vortex mixer for 30 seconds and centrifuged for 5 minutes at 4000 r.p.m. An aliquot of benzene (upper) phase was injected into a Tracor 222 gas chormatograph equipped with a $Ni^{63}$ electron capture detection system.

2.9.4 Gas Chromatographic Analysis - Catechol and Hydroquinone

A standard curve was generated by injecting known amounts of combined catechol and hydroquinone (0.050 - 0.50 ng). Standards were plotted as peak height vs. ng injected. Residues of catechol and hydroquinone were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 8, 9 and 12, respectively. Gas chromatographic conditions are given in Table VI. The limit of detection is 0.5 ppm.

## 2.10 Determination of Conjugated Phenol, Catechol and Hydroquinone in Rat Blood by Gas Chromatographic Analysis

### 2.10.1 Sample Preparation - Phenol, Catechol and Hydroquinone

An enzyme/buffer solution was prepared by combining 15 ml of pH 5 sodium acetate buffer with 0.018 g of EDTA and 1.5 g of β-glucuronidase (1.0 million units per 2.5 g). A 0.75 ml of this solution was added to the 1 ml aliquot of blood previously extracted for free phenol, catechol and hydroquinone. The mixture was vortexed well and incubated overnight at 37°C.

After incubation, 2 ml of normal saline were added to the samples and the mixture was extracted three times with 3 ml portions of ethyl acetate. Each extraction was centrifuged at 4000 r.p.m. for 10 minutes, and the ethyl acetate (upper) phases were combined and centrifuged. The combined extract was divided into a 6 ml aliquot for phenol analysis and a 3 ml aliquot for combined catechol and hydroquinone analysis.

For phenol analysis, the 6 ml aliquot of blood extract was evaporated to 0.5 ml under dry nitrogen and transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

For catechol and hydroquinone analysis, the 3 ml aliquot of blood extract was evaporated almost to dryness with dry nitrogen. The residue was caused to react with 200 ul of TFAA for 30 minutes at 50°C. Two ml of benzene and 5.0 ml of

pH 6 phosphate buffer were added to the derivative solution, the solution was mixed on a Vortex mixer for 30 seconds and centrifuged for 5 minutes at 4000 r.p.m. An aliquot of the benzene (upper) phase was injected into a Tracor 222 gas chromatograph equipped with a $Ni^{63}$ electron capture detection system.

2.10.2 Gas Chromatographic Analysis - Phenol

A standard curve was generated by injecting known amounts of phenol (1.0 - 40.0 ng). Standards are plotted as the peak height of phenol vs. ng of phenol injected. Residues of phenol were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 5, 6 and 13, respectively. Gas chromatographic conditions are given in Table V. The limit of detection is 1.0 ppm.

2.10.3 Gas Chromatographic Analysis - Catechol and Hydroquinone

A standard curve was generated by injecting known amounts of combined catechol and hydroquinone (0.050 - 0.50 ng). Standards were plotted as peak height vs. ng injected. Residues of catechol and hydroquinone were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 8, 9 and 14, respectively. Gas chromatographic conditions are given in Table VI. The limit of detection is 0.5 ppm.

## 2.11 Determination of Conjugated Phenol, Catechol and Hydroquinone in Mouse Blood by Gas Chromatographic Analysis

### 2.11.1 Sample Preparation - Phenol, Catechol and Hydroquinone

An enzyme/buffer solution was prepared by combining 15 ml of pH 5 sodium acetate buffer with 0.018 g of EDTA and 1.5 g of $\beta$-glucuronidase (1.0 million units per 2.5 g). A 0.75 ml of this solution was added to the 0.5 ml aliquot of blood previously extracted for free phenol, catechol and hydroquinone. The mixture was vortexed well and incubated overnight at 37°C.

After incubation, 2 ml of normal saline were added to the samples and the mixture was extracted three times with 3 ml portions of ethyl acetate. Each extraction was centrifuged at 4000 r.p.m. for 10 minutes, and the ethyl acetate (upper) phases were combined and centrifuged. The combined extract was divided into a 6 ml aliquot for phenol analysis and a 3 ml aliquot for combined catechol and hydroquinone analysis.

For phenol analysis, the 6 ml aliquot of blood extract was evaporated to 0.25 ml under dry nitrogen and transferred to a vial for subsequent analysis on a Tracor 550 gas chromatograph equipped with a flame ionization detection system.

For catechol and hydroquinone analysis, the 3 ml aliquot of blood extract was evaporated almost to dryness with dry nitrogen. The residue was caused to react with 200 ul of TFAA for 30 minutes at 50°C. Two ml of benzene and 5.0 ml of pH 6

phosphate buffer were added to the derivative solution, the solution was mixed on a Vortex mixer for 30 seconds and centrifuged for 5 minutes at 4000 r.p.m. An aliquot of the benzene (upper) phase was injected into a Tracor 222 gas chromatograph equipped with a $Ni^{63}$ electron capture detection system.

2.11.2 Gas Chromatographic Analysis - Phenol

A standard curve was generated by injecting known amounts of phenol (1.0 - 20.0 ng). Standards were plotted as the peak height of phenol vs. ng of phenol injected. Residues of phenol were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 5, 16 and 15, respectively. Gas chromatographic conditions are given in Table V. The limit of detection is 1.0 ppm.

2.11.3 Gas Chromatographic Analysis - Catechol and Hydroquinone

A standard curve was generated by injecting known amounts of combined catechol and hydroquinone (0.050 - 0.50 ng). Standard are plotted as peak height vs. ng injected. Residues of catechol and hydroquinone were determined by comparing peak heights of samples with the standard curve. A typical standard curve and examples of standard and sample injections are given in Figures 8, 9 and 16, respectively. Gas chromatographic conditions are given in Table VI. The limit of detection is 0.5 ppm.

## 3.0 RECOVERY DATA

Procedural recoveries were determined for benzene, phenol, catechol and hydroquinone by fortifying control rat and mouse blood with known amounts of compound, and carrying these samples through the procedure. Determinations were made on the appropriate chromatogarphic system, and the values obtained were compared to the nominal concentration (ppm). The average recoveries of free benzene, phenol, catechol and hydroquinone in rat blood were 114, 97, 99 and 93%, respectively. The average recoveries of free benzene, phenol, catechol and hydroquinone in mouse blood were 148, 109, 106 and 92%, respectively.

Packed red blood cells from control rats and mice which were previously extracted for free benzene and metabolites, were also spiked with known amounts of compound, incubated, worked-up and analyzed according to the analytical methodology for conjugates. The average recoveries of phenol, catechol and hydroquinone were 103, 90 and 94%, respectively.

Complete recovery data are summarized in Tables VII, VIII and IX. Sample assays were not corrected for procedural recovery.

## 4.0 RESULTS AND DISCUSSION

### 4.1 Benzene Concentrations

The exposed rats received exposure to a mean chamber atmosphere concentration of 301 ppm (S.D. = 10 ppm) for

the 12 hour exposure period. The treated mice received exposure to a mean chamber concentration of 296 ppm (S.D. = 15 ppm), based upon infrared analysis.

4.2 Animal Results

No abnormalities were observed in any of the treated or control rats or mice at any bleeding interval. The time elapsed from removal to orbital bleeding was approximately two minutes per rat. Approximately nine minutes were required to complete blood sample collection from all mice in a group due to the number of animals involved.

4.3 Levels of Benzene and Metabolites in Rodent Blood

The levels of benzene in rat blood ranged from 3.7 to 5.2 ppm at one hour to 6.0 to 8.3 ppm at 12 hours. Outside of 2 samples (apparently artifacts due to relatively high level) no detectable levels of free phenol, catechol and hydroquinone were observed. A complete summary of these data is given in Table X.

The levels of benzene in mouse blood ranged from 3.5 to 3.9 ppm at one hour to 1.3 to 1.4 ppm at 12 hours. The levels of free phenol in mouse blood ranged from 1.8 to 2.9 ppm at one hour to 1.8 to 2.6 ppm at 12 hours. Levels of free phenol were observed in the 0 and 12 hour control mice. These levels ranged from 1.9 to 2.1 ppm at 0 hour to 2.3 to 2.5 ppm at 12 hours. These residues may be due to naturally occurring phenol in the blood. No detectable levels of free catechol and hydroquinone were seen. A summary of these data is given in Table XI.

The levels of conjugated phenol in rat blood ranged from <1.0 to 6.0 ppm at one hour to 4.0 to 8.5 ppm at 12 hours. Two phenol residues were observed in 0 and 12 hour control rats of 2.1 and 1.2 ppm, respectively. (These residues were probably artifacts due to the unusually high levels.) No detectable levels of conjugated catechol and hydroquinone were seen at any of the intervals assayed. These data are summarized in Table XII.

The levels of conjugated phenol in mouse blood ranged from 5.9 to 9.2 ppm at one hour to 3.4 to 4.0 ppm at 12 hours. Phenol was detected in the blood of control animals at 0 and 12 hours. These levels may be attributed to artifacts or naturally occurring phenol in the blood. Catechol and hydroquinone were not detected. A summary of these data is given in Table XIII. The mean levels of benzene and its metabolites in rat blood are depicted in Figures 17-19.

These data indicate the time to steady-state level in rat and mouse blood is approximately one hour. Animals for Phase II of this study will be exposed to benzene at varying concentrations for six hours.

TABLE I. PURITY ANALYSIS OF BENZENE USED FOR STUDY NO. 79-7240

| Analysis | Results | Method |
|---|---|---|
| Freezing Point | 5.433°C | ASTM D-1015 |
| Freezing Point Purity (calculated) | 99.85 ± 0.02 mole % | D-1016 |
| Total Sulfur | 1 ppm | ASTM D-3246-76 and AMS 60.23 |
| Toluene<br>Non-aromatics<br>Unknown hydrocarbon | 68 ppm )<br>22 ppm )<br>2 ppm ) | AMI 710.79 |
| Specific Gravity @ 60°F | 0.8845 | BRCP 8615 |

TABLE II. COMPARISON OF METHODS FOR THE EXTRACTION OF $^{14}C$-LABELED BENZENE AND PHENOL FROM WHOLE RAT BLOOD

Benzene

| | % $^{14}C$-Activity | |
|---|---|---|
| Experiment | C.I.I.T. Method[a] | Modified C.I.I.T. Method[b] |
| A | 21.7 | 84.1 |
| B | 22.7 | 75.3 |
| C | 0.7 | 81.2 |
| D | 1.5 | 63.1 |
| E | 51.9 | 88.7 |
| F | - | 83.3 |
| G | - | 90.2 |

a. One pass extraction of whole blood with an equal volume of methanol.
b. One pass extraction of whole blood with an equal volume of methylene chloride.

Phenol

| | % $^{14}C$-Activity | |
|---|---|---|
| Experiment | C.I.I.T. Method[a] | Modified C.I.I.T. Method[b] |
| A | 20.1 | 89.3 |
| B | 28.0 | 91.1 |
| C | 17.7 | 91.2 |
| D | - | 84.9 |

a. One pass extraction of whole blood with an equal volume of methanol.
b. Extraction of whole blood three times with an equal volume of diethyl ether.

TABLE III. SUMMARY OF EXPERIMENTAL CONDITIONS EMPLOYED TO DETERMINE SULFATE AND β-GLUCURONIDE CONJUGATES OF PHENOLS

| Experiment No. | Experimental Conditions | Amount of Enzyme (Units) | | % Phenol Liberated | |
|---|---|---|---|---|---|
| | | β-Glucuronidase[a] | Sulfatase[b] | Estradiol | Estrone |
| 1 | Freeze-dried RBCs | 7,600 | 1,860 | 34 | 87 |
| 2 | RBCs | 7,600 | 1,860 | 60 | 54 |
| 3 | RBCs | 30,000 | 3,000 | 78 | 77 |
| 4 | RBCs | 60,000 | 6,000 | 65 | 61 |

a. 1 unit of enzyme will liberate 1 ug of phenolphthalein from phenolphthalein glucuronide per hour at 37°C at pH 5.

b. 1 g of enzyme will hydrolyze 40,000 u moles of p-nitrocatechol sulfate per our at pH 5 at 37°C.

TABLE IV. GAS CHROMATOGRAPHIC CONDITIONS FOR BENZENE IN RAT AND MOUSE BLOOD

| | |
|---|---|
| Column: | 6' x 4 mm i.d., glass |
| Liquid Phase: | 0.2% Carbowax 1500 |
| Support: | Carbopack C |
| Mesh Size: | 80/100 |
| Column Temperature: | 110°C |
| Detector Temperature: | 275°C |
| Injection Port Temperature: | 240°C |
| Column Flow Rate: | 25 ml/min. |
| $H_2$ Flow Rate: | 25 ml/min. |
| Air Flow Rate: | 1.0 SCFH |
| Carrier Gas: | $N_2$ |

TABLE V. GAS CHROMATOGRAPHIC CONDITIONS FOR FREE PHENOL IN RAT AND MOUSE BLOOD

| | |
|---|---|
| Column: | 6' x 2 mm i.d., glass |
| Liquid Phase: | 1.0% SP-1240 DA |
| Support: | Supelcoport |
| Mesh Size: | 100/120 |
| Column Temperature: | 90°C |
| Detector Temperature: | 275°C |
| Injection Port Temperature: | 240°C |
| Column Flow Rate: | 25 ml/min. |
| $H_2$ Flow Rate: | 25 ml/min. |
| Air Flow Rate: | 1.0 SCFH |
| Carrier Gas: | $N_2$ |

TABLE VI. GAS CHROMATOGRAPHIC CONDITIONS FOR FREE CATECHOL AND HYDROQUINONE IN RAT AND MOUSE BLOOD

| | |
|---|---|
| Column: | 6' x 2 mm i.d., glass |
| Liquid Phase: | 5% OV-1 |
| Support: | GCQ |
| Mesh Size: | 80/100 |
| Column Temperature: | 70°C |
| Detector Temperature: | 320°C |
| Injection Port Temperature: | 270°C |
| Column Flow Rate: | 50 ml/min. |
| Purge Flow Rate: | 10 ml/min. |
| Carrier Gas: | 5% methane/argon |

TABLE VII. RECOVERY OF BENZENE, FREE PHENOL, CATECHOL AND HYDROQUINONE FROM WHOLE BLOOD FOR PHASE I - RAT STUDY

| Compound | Residue, ppm Added | Residue, ppm Found | % Recovery | Average Recovery, % |
|---|---|---|---|---|
| Benzene | 1.0 | 1.1 | 110 | |
| | 5.0 | 6.0 | 120 | |
| | 10.0 | 11.5 | 115 | 114 |
| | 20.0 | 23.0 | 115 | |
| | 30.0 | 33.6 | 112 | |
| Phenol | 1.0 | 0.97 | 97 | |
| | 5.0 | 5.0 | 100 | |
| | 10.0 | 9.6 | 96 | 97 |
| | 20.0 | 19.5 | 97 | |
| | 30.0 | 27.6 | 92 | |
| Catechol | 1.0 | 1.06 | 106 | |
| | 5.0 | 5.03 | 101 | |
| | 10.0 | 10.45 | 105 | |
| | 20.0 | 21.09 | 105 | 99 |
| | 30.0 | 28.79 | 96 | |
| | 0.50 | 0.447 | 89 | |
| | 1.0 | 0.936 | 94 | |
| Hydroquinone | 1.0 | 0.79 | 79 | |
| | 5.0 | 4.47 | 89 | |
| | 10.0 | 9.24 | 92 | |
| | 20.0 | 19.09 | 95 | 93 |
| | 30.0 | 26.14 | 87 | |
| | 0.10 | 0.103 | 103 | |
| | 0.50 | 0.503 | 101 | |
| | 1.0 | 1.02 | 102 | |

TABLE VIII. RECOVERY OF BENZENE, FREE PHENOL, CATECHOL AND HYDROQUINONE FROM WHOLE BLOOD FOR PHASE I - MOUSE STUDY

| Compound | Residue, ppm Added | Residue, ppm Found | % Recovery | Average Recovery, % |
|---|---|---|---|---|
| Benzene | 1.00 | 1.60 | 160 | |
| | 2.50 | 3.75 | 150 | 148 |
| | 5.00 | 8.15 | 163 | |
| | 10.00 | 11.75 | 118 | |
| Phenol: | 1.00* | 1.20 | 120 | |
| | 5.00* | 4.96 | 99 | 109 |
| | 10.0 * | 10.68 | 107 | |
| Catechol: | 0.500 | 0.536 | 107 | |
| | 1.000 | 1.132 | 113 | 105 |
| | 2.000 | 1.928 | 96 | |
| Hydroquinone | 0.500 | 0.494 | 99 | |
| | 1.000 | 1.024 | 102 | 92 |
| | 2.000 | 1.473 | 74 | |

*Taken from 5.8.79 exposure.

TABLE IX. RECOVERY OF CONJUGATED PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I RAT AND MOUSE STUDIES

| Compound | Residue, ppm Added | Residue, ppm Found | % Recovery | Average Recovery, % |
|---|---|---|---|---|
| Phenol | 1.00 | 1.05 | 105 | |
| | 5.00 | 5.10 | 102 | |
| | 10.00 | 7.35 | 74 | 103 |
| | 1.00 | 1.05 | 105 | |
| | 5.00 | 5.55 | 111 | |
| | 10.00 | 12.14 | 121 | |
| Catechol | 0.50 | 0.45 | 90 | 90 |
| Hydroquinone | 0.50 | 0.47 | 94 | 94 |

TABLE X. SUMMARY OF RESIDUES OF BENZENE, FREE PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I - RAT STUDY

| Animal No. | Interval (Hr) | Residue, ppm | | | |
|---|---|---|---|---|---|
| | | Benzene | Phenol | Catechol | Hydroquinone |
| 40 | 0 | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 41 | 0 | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 42 | 0 | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 43 | 0 | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 44 | 1 | 3.7 | < 1.0 | < 0.50 | < 0.10 |
| 45 | 1 | 4.6 | < 1.0 | < 0.50 | < 0.10 |
| 46 | | 5.1 | < 1.0 | < 0.50 | < 0.10 |
| 47 | 1 | 5.2 | < 1.0 | < 0.50 | < 0.10 |
| Mean | | 4.7 | | | |
| 48 | 2 | 4.9 | < 1.0 | < 0.50 | < 0.10 |
| 49 | 2 | 4.3 | < 1.0 | < 0.50 | < 0.10 |
| 50 | 2 | 5.1 | < 1.0 | < 0.50 | < 0.10 |
| 51 | 2 | 4.7 | < 1.0 | < 0.50 | < 0.10 |
| Mean | | 4.7 | | | |
| 52 | 4 | 5.6 | < 1.0 | < 0.50 | < 0.10 |
| 53 | 4 | 5.4 | < 1.0 | < 0.50 | < 0.10 |
| 54 | 4 | 5.9 | < 1.0 | < 0.50 | < 0.10 |
| 55 | 4 | 5.8 | < 1.0 | < 0.50 | < 0.26 |
| Mean | | 5.7 | | | |
| 56 | 8 | 4.8 | < 1.0 | < 0.50 | < 0.10 |
| 57 | 8 | 6.0 | < 1.0 | < 0.50 | < 0.10 |
| 58 | 8 | 3.4 | < 1.0 | < 0.50 | < 0.10 |
| 59 | 8 | 7.1 | < 1.0 | < 0.50 | < 0.10 |
| Mean | | 5.3 | | | |
| 60 | 12 | 7.1 | < 1.0 | < 0.50 | < 0.10 |
| 61 | 12 | 6.0 | < 1.0 | < 0.50 | < 0.10 |
| 62 | 12 | 8.3 | < 1.0 | < 0.50 | < 0.10 |
| 63 | 12 | 6.8 | < 1.0 | < 0.50 | 2.15 |
| Mean | | 7.0 | | | |
| 64 | 12 Control | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 65 | 12 Control | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 66 | 12 Control | < 1.0 | < 1.0 | < 0.50 | < 0.10 |
| 67 | 12 Control | < 1.0 | < 1.0 | < 0.50 | < 0.10 |

Note: Mean values are calculated by omitting < figures and dividing the sum of actual observed figures by the number of data points per exposure interval.

TABLE XI. SUMMARY OF RESIDUES OF BENZENE, FREE PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I - MOUSE STUDY

| Animal No. | Interval (Hr) | Residue, ppm | | | |
|---|---|---|---|---|---|
| | | Benzene | Phenol | Catechol | Hydroquinone |
| 600, 601<br>602, 603 | 0 | < 1.0 | 1.88 | < 0.500 | < 0.500 |
| 604, 605<br>606, 607 | 0 | < 1.0 | 1.88 | < 0.500 | < 0.500 |
| 608, 609<br>610, 611 | 0 | < 1.0 | 2.11 | < 0.500 | < 0.500 |
| 612, 613<br>614, 615 | 0 | < 1.0 | 2.11 | < 0.500 | < 0.500 |
| Mean | | | 1.99 | | |
| 616, 617<br>618, 619 | 1 | 3.55 | 1.80 | < 0.500 | < 0.500 |
| 620, 621<br>622, 623 | 1 | 3.75 | 2.48 | < 0.500 | < 0.500 |
| 624, 625<br>626, 627 | 1 | 3.90 | 2.86 | < 0.500 | < 0.500 |
| 628, 629<br>630, 631 | 1 | 3.50 | 2.48 | < 0.500 | < 0.500 |
| Mean | | 3.67 | 2.41 | | |
| 632, 633<br>634, 635 | 2 | 2.50 | 2.30 | < 0.500 | < 0.500 |
| 636, 637<br>638, 639 | 2 | 3.40 | 2.30 | < 0.500 | < 0.500 |
| 640, 641<br>642, 643 | 2 | 2.80 | 2.00 | < 0.500 | < 0.500 |
| 644, 645<br>646, 647 | 2 | 3.40 | 2.00 | < 0.500 | < 0.500 |
| Mean | | 3.03 | 2.15 | | |
| 648, 649<br>650, 651 | 4 | 2.60 | 2.40 | < 0.500 | < 0.500 |
| 652, 653<br>654, 655 | 4 | 2.50 | 2.00 | < 0.500 | < 0.500 |
| 656, 657<br>658, 659 | 4 | 2.25 | 2.40 | < 0.500 | < 0.500 |
| 660, 661<br>662, 663 | 4 | 2.25 | 2.30 | < 0.500 | < 0.500 |
| Mean | | 2.40 | 2.27 | | |

TABLE XI. SUMMARY OF RESIDUES OF BENZENE, FREE PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I - MOUSE STUDY - continued

| Animal No. | Interval (Hr) | Residue, ppm | | | |
|---|---|---|---|---|---|
| | | Benzene | Phenol | Catechol | Hydroquinone |
| 664, 665 666, 667 | 8 | 2.45 | 2.50 | < 0.500 | < 0.500 |
| 668, 669 670, 671 | 8 | 3.60 | 2.40 | < 0.500 | < 0.500 |
| 672, 673 674, 675 | 8 | 3.50 | 2.30 | < 0.500 | < 0.500 |
| 676, 677 678, 679 | 8 | 2.60 | 2.80 | < 0.500 | < 0.500 |
| Mean | | 3.04 | 2.50 | | |
| 680, 681 682, 683 | 12 | 1.38 | 2.30 | < 0.500 | < 0.500 |
| 684, 685 686, 687 | 12 | 1.26 | 1.80 | < 0.500 | < 0.500 |
| 688, 689 690, 691 | 12 | 1.38 | 2.30 | < 0.500 | < 0.500 |
| 692, 693 694, 695 | 12 | 1.26 | 2.60 | < 0.500 | < 0.500 |
| Mean | | 1.32 | 2.25 | | |
| 696, 697 698, 699 | 12 Control | < 1.0 | 2.50 | < 0.500 | < 0.500 |
| 700, 701 702, 703 | 12 Control | < 1.0 | 2.50 | < 0.500 | < 0.500 |
| 704, 705 706, 707 | 12 Control | < 1.0 | 2.30 | < 0.500 | < 0.500 |
| 708, 709 710, 711 | 12 Control | < 1.0 | 2.40 | < 0.500 | < 0.500 |
| Mean | | | 2.43 | | |

Note: Mean values are calculated by omitting < figures and dividing the sum of actual observed figures by the number of data points per exposure interval.

TABLE XII. SUMMARY OF RESIDUES OF CONJUGATED PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I - RAT STUDY

| Animal No. | Interval (Hr) | Residue, ppm | | |
|---|---|---|---|---|
| | | Phenol | Catechol | Hydroquinone |
| 40 | 0 | < 1.00 | <0.50 | < 0.50 |
| 41 | 0 | < 1.0 | <0.50 | < 0.50 |
| 42 | 0 | 2.1 | <0.50 | < 0.50 |
| 43 | 0 | < 1.00 | <0.50 | < 0.50 |
| Mean | | < 1.00 | | |
| 44 | 1 | 5.92 | - | - |
| 45 | 1 | 1.00 | - | - |
| 46 | 1 | 3.00 | - | - |
| 47 | 1 | 2.92 | - | - |
| Mean | | 2.96 | | |
| 48 | 2 | 4.00 | <0.50 | < 0.50 |
| 49 | 2 | 4.90 | <0.50 | < 0.50 |
| 50 | 2 | 6.10 | - | - |
| 51 | 2 | 6.10 | - | - |
| Mean | | 5.28 | | |
| 52 | 4 | 3.56 | <0.50 | < 0.50 |
| 53 | 4 | 5.10 | <0.50 | < 0.50 |
| 54 | 4 | 5.02 | <0.50 | < 0.50 |
| 55 | 4 | 3.26 | <0.50 | < 0.50 |
| Mean | | 4.24 | | |
| 56 | 8 | 6.90 | <0.50 | < 0.50 |
| 57 | 8 | 6.80 | <0.50 | < 0.50 |
| 58 | 8 | 8.50 | - | - |
| 59 | 8 | 6.30 | - | - |
| Mean | | 7.13 | | |
| 60 | 12 | 4.01 | <0.50 | < 0.50 |
| 61 | 12 | 4.61 | | - |
| 62 | 12 | 1.61 | - | - |
| 63 | 12 | 8.47 | - | - |
| Mean | | 4.68 | | |
| 64 | 12 Control | 1.20 | <0.50 | < 0.50 |
| 65 | 12 Control | < 1.00 | <0.50 | < 0.50 |
| 66 | 12 Control | < 1.00 | - | - |
| 67 | 12 Control | < 1.00 | - | - |
| Mean | | < 1.00 | | |

Note: Mean values are calculated by omitting < figures and dividing the sum of actual observed figures - by the number of data points per exposure interval.

TABLE XIII. SUMMARY OF RESIDUES OF CONJUGATED PHENOL, CATECHOL AND HYDROQUINONE FOR PHASE I - MOUSE STUDY

| Animal No. | Interval (Hr) | Residue, ppm | | |
|---|---|---|---|---|
| | | Phenol | Catechol | Hydroquinone |
| 600-603 | 0 | 1.50 | < 0.50 | < 0.50 |
| 604-607 | 0 | 2.77 | < 0.50 | < 0.50 |
| 608-611 | 0 | 6.60 | < 0.50 | < 0.50 |
| 612-615 | 0 | < 1.00 | < 0.50 | < 0.50 |
| Mean | | 2.71 | | |
| 616-619 | 1 | 0.15 | < 0.50 | < 0.50 |
| 620-623 | 1 | 5.85 | < 0.50 | < 0.50 |
| 624-627 | 1 | 7.72 | < 0.50 | < 0.50 |
| 628-631 | 1 | 6.07 | < 0.50 | < 0.50 |
| Mean | | 7.20 | | |
| 632-635 | 2 | 6.60 | < 0.50 | < 0.50 |
| 636-639 | 2 | 7.42 | < 0.50 | < 0.50 |
| 640-643 | 2 | 10.72 | < 0.50 | < 0.50 |
| 644-647 | 2 | 10.12 | < 0.50 | < 0.50 |
| Mean | | 8.71 | | |
| 648-651 | 4 | 10.42 | < 0.50 | < 0.50 |
| 652-655 | 4 | 7.95 | < 0.50 | < 0.50 |
| 656-659 | 4 | 5.85 | < 0.50 | < 0.50 |
| 660-663 | 4 | 9.30 | < 0.50 | < 0.50 |
| Mean | | 8.38 | | |
| 664-667 | 8 | 6.30 | < 0.50 | < 0.50 |
| 668-671 | 8 | 7.50 | < 0.50 | < 0.50 |
| 672-675 | 8 | 5.40 | < 0.50 | < 0.50 |
| 676-679 | 8 | 17.20 | < 0.50 | < 0.50 |
| Mean | | 9.10 | | |
| 680-683 | 12 | 3.40 | < 0.50 | < 0.50 |
| 684-687 | 12 | 3.40 | < 0.50 | < 0.50 |
| 688-691 | 12 | 4.00 | < 0.50 | < 0.50 |
| 692-695 | 12 | 3.90 | < 0.50 | < 0.50 |
| Mean | | 3.68 | | |
| 696-699 | 12 Control | 4.80 | < 0.50 | < 0.50 |
| 700-703 | 12 Control | < 1.00 | < 0.50 | < 0.50 |
| 704-707 | 12 Control | < 1.00 | < 0.50 | < 0.50 |
| 708-711 | 12 Control | 1.90 | < 0.50 | < 0.50 |
| Mean | | 1.67 | | |

Note: Mean values are calculated by omitting < figures and dividing the sum of actual observed figures by the number of data points per exposure interval.

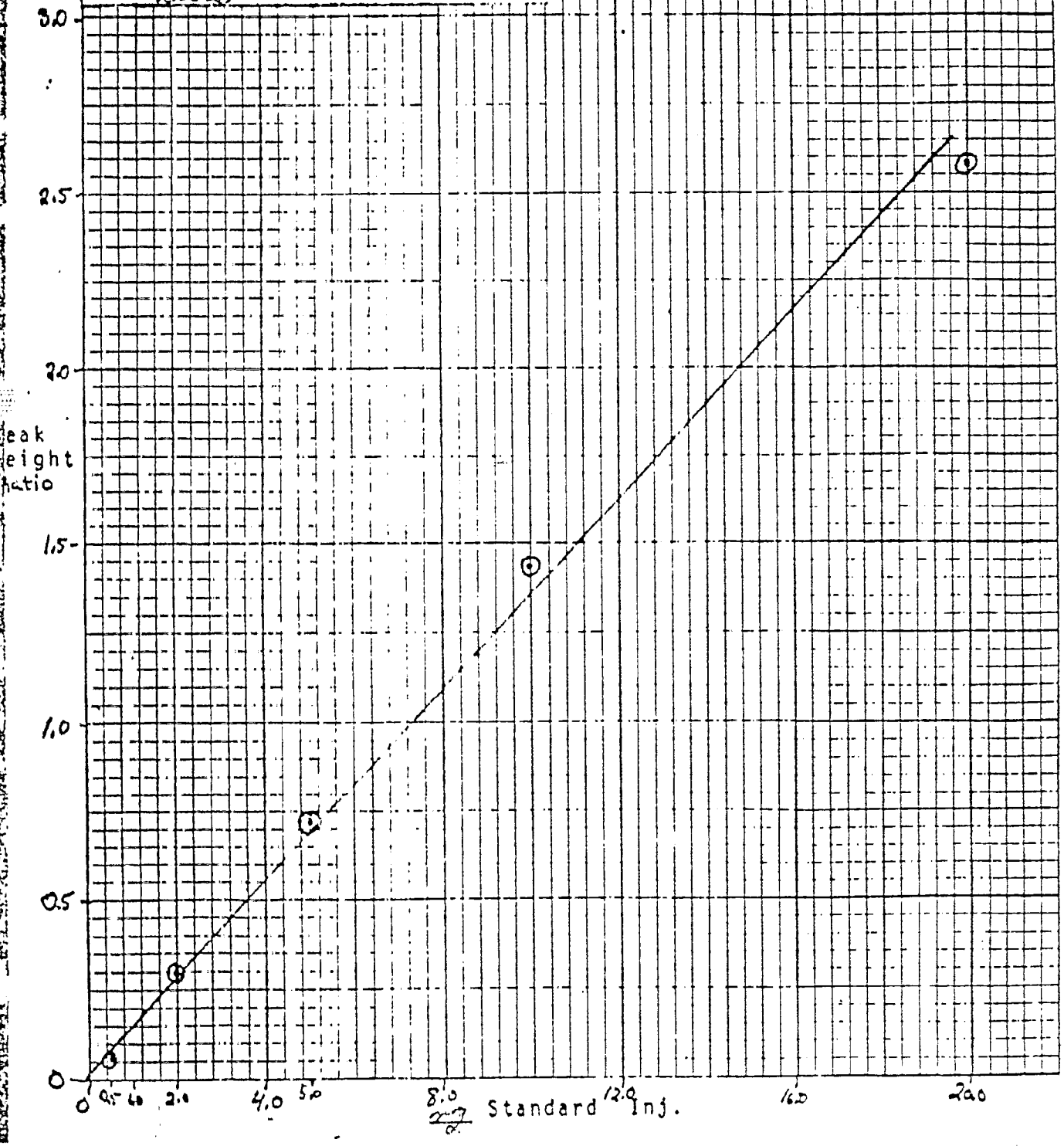
TABLE XIV. CONDITIONS OF ANIMAL HUSBANDRY

| | |
|---|---|
| Lights: | 12 hours on, 12 hours off |
| During Exposure Period | |
| Housing: | Groups of 4 mice or 2 rats in stainless steel wire mesh cages |
| Food: | None |
| Water: | None |
| During Pre-Exposure Period | |
| Housing: | Paired in hung stainless steel wire mesh bottom cages |
| Food: | Standard rat chow - ad libitum |
| Water: | Tap-City water with automatic watering system - ad libitum |
| Identification: | Individual ear tag after selection |
| Equilibration Period: | Minimum 12 days |
| Age on Test: | 7 weeks |

Operator R. Minchin Date 4-2-79
Instrument # TRACOR Detector FID
Column: Flow Rates:
Length 6 ft. Hydrogen 30 Air 1.0
I.D. 4 mm. Scavenge
Liquid Phase CARBOWAX 1500 Split
Wt. % 0.2 Pressure psig
Lot # Temperatures:
Support CARBOPAK C. Column 110 °C
Mesh 80/100 Detector 210 °C
Carrier Gas N2 FH 240 °C
Rate 25 ml/min. Sensitivity 4X1
Chart Speed 0.2 in/min. Cell Voltage
Sample: Blood Sample Size 1.0A

E.A.C. Project No. 79004
Study No.
Compound BENZENE

FIGURE 1. BENZENE STANDARD CURVE




DBZ0020383

DB2002038 4



FIGURE 2. TYPICAL BENZENE/FLUOROBENZENE STANDARD CHROMATOGRAM

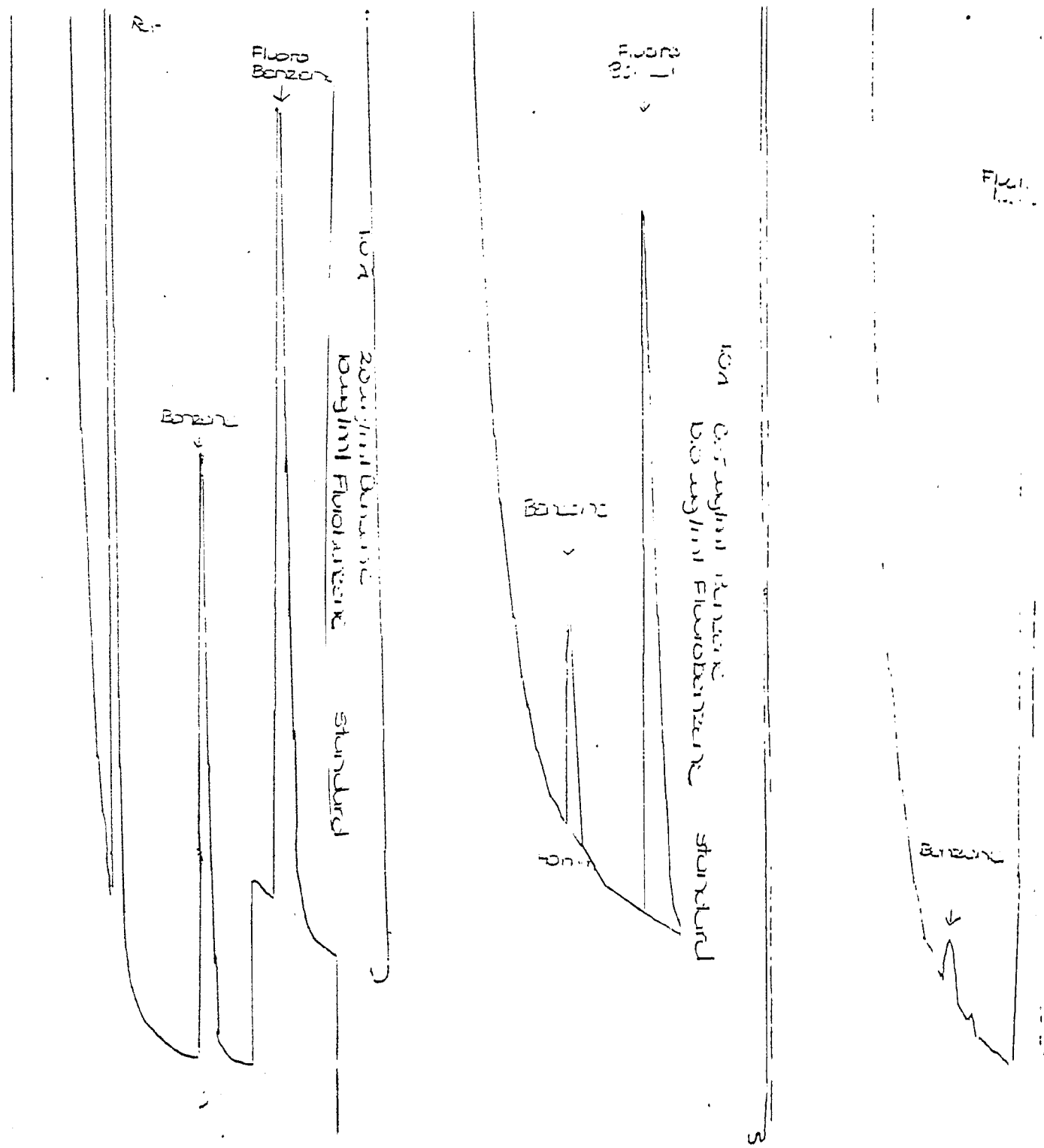


FIGURE 3. TYPICAL CHROMATOGRAM OF BENZENE IN RAT BLOOD EXTRACT

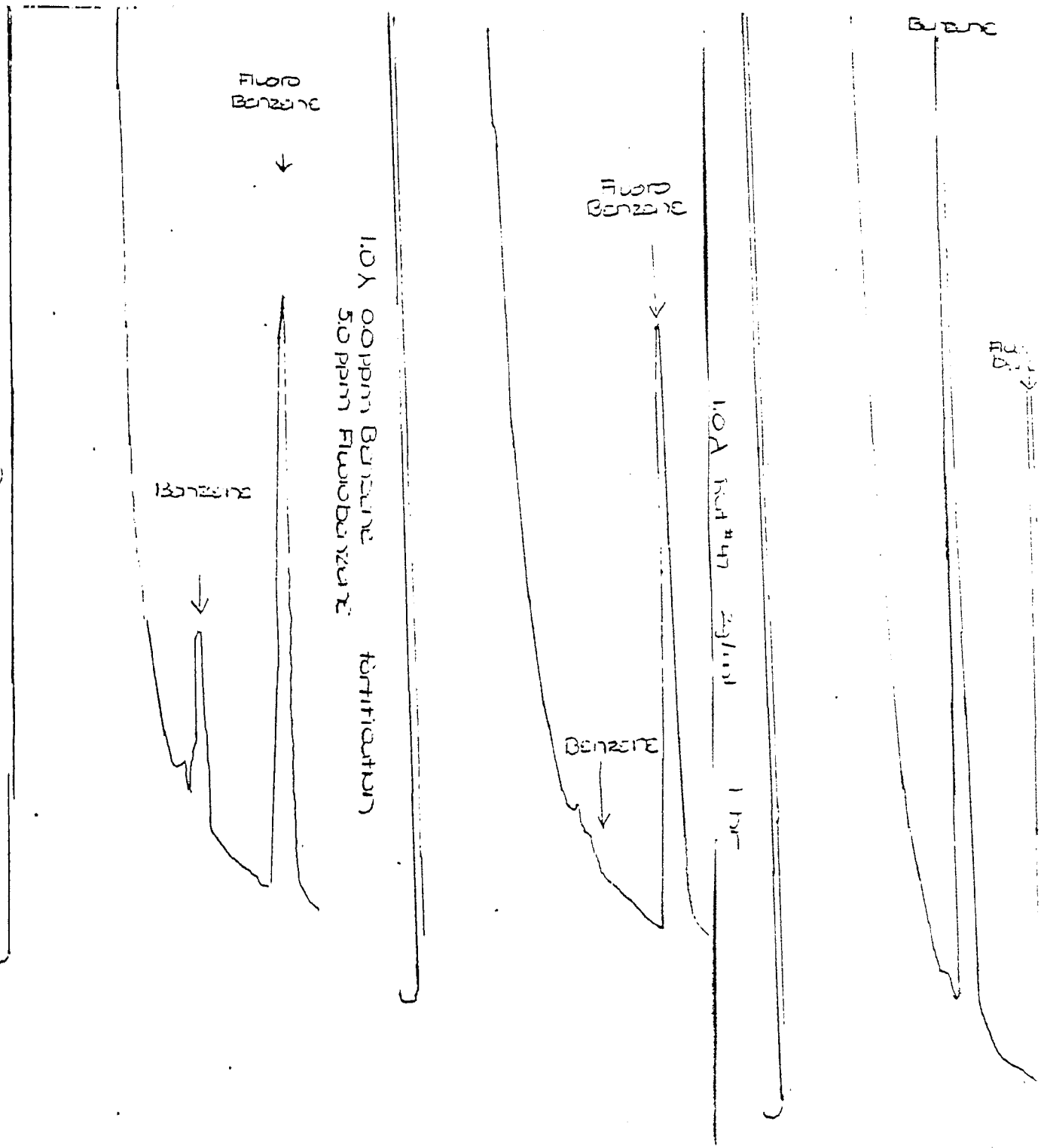
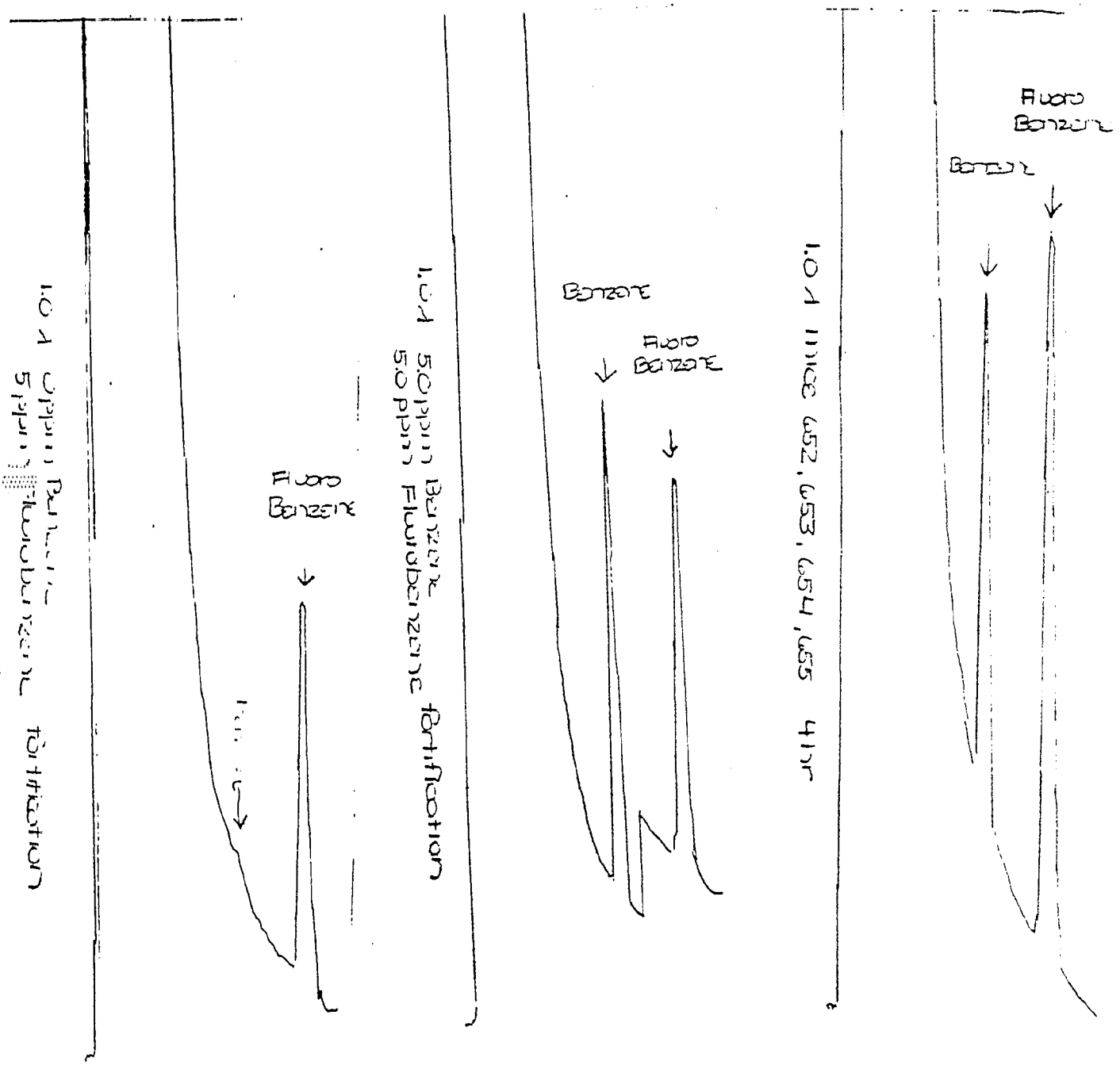
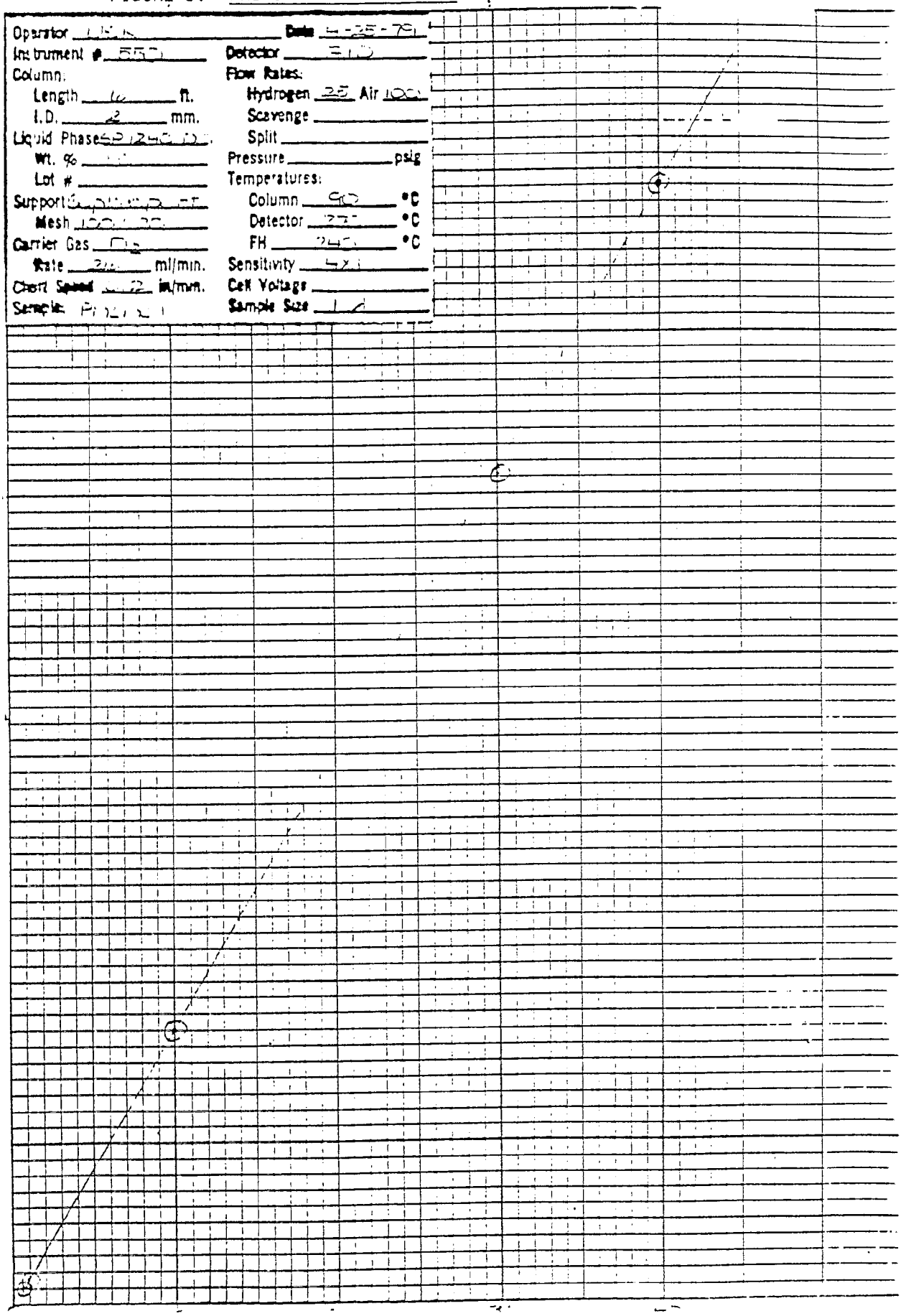


FIGURE 4. TYPICAL CHROMATOGRAM OF BENZENE IN MOUSE BLOOD EXTRACT



EK Project No 79004
Study No
Compound: Phenol

FIGURE 5. PHENOL STANDARD CURVE




46 0622

KE 10 X 10 TO 1-1/4 INCH • 7 X 10 INCHES
KEUFFEL & ESSER CO MADE IN U.S.A.

DBZ0020387

FIGURE 6. TYPICAL PHENOL/p-CRESOL STANDARD CHROMATOGRAM

790344

FIGURE 7. TYPICAL CHROMATOGRAM OF PHENOL IN RAT BLOOD EXTRACT

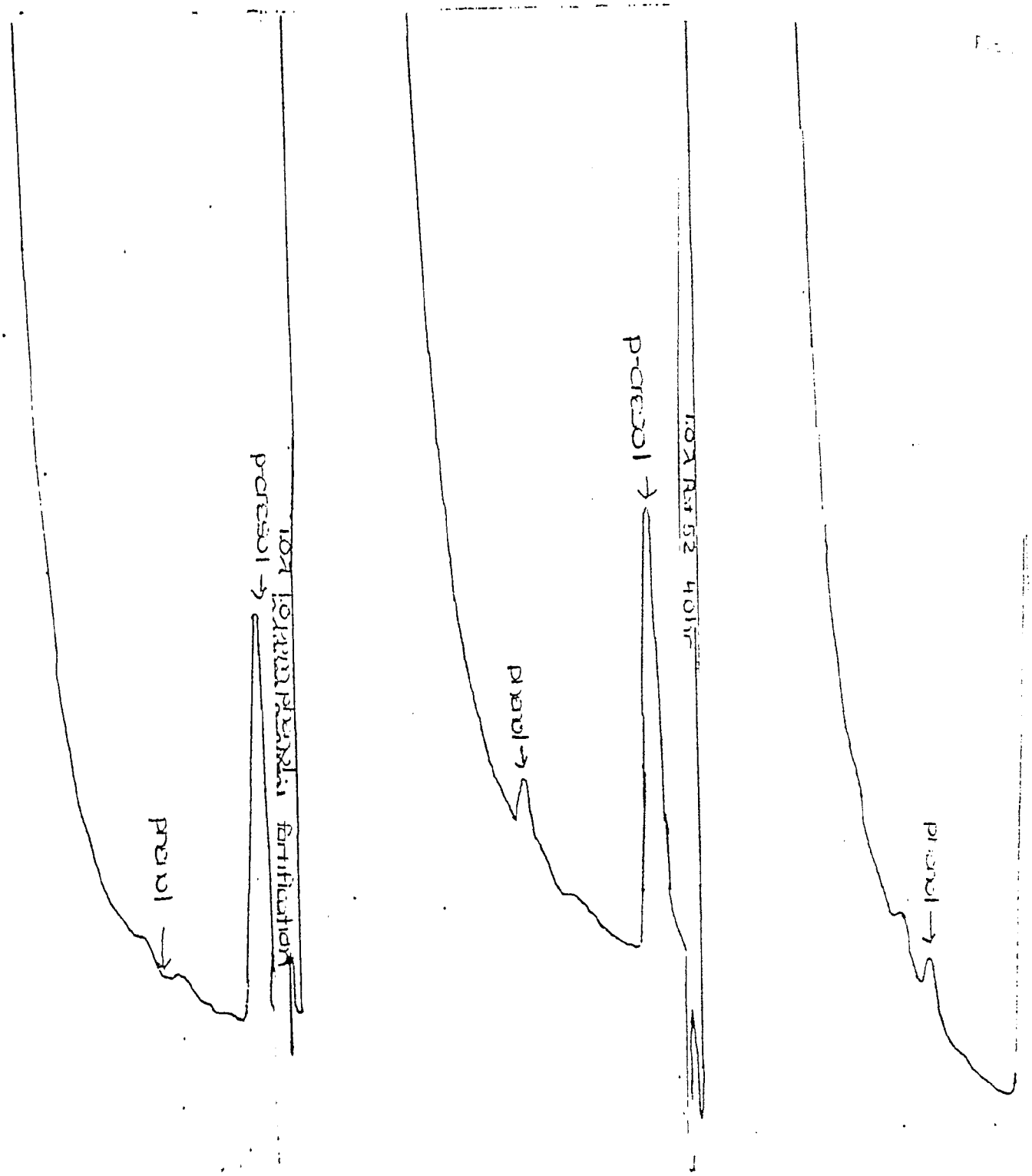
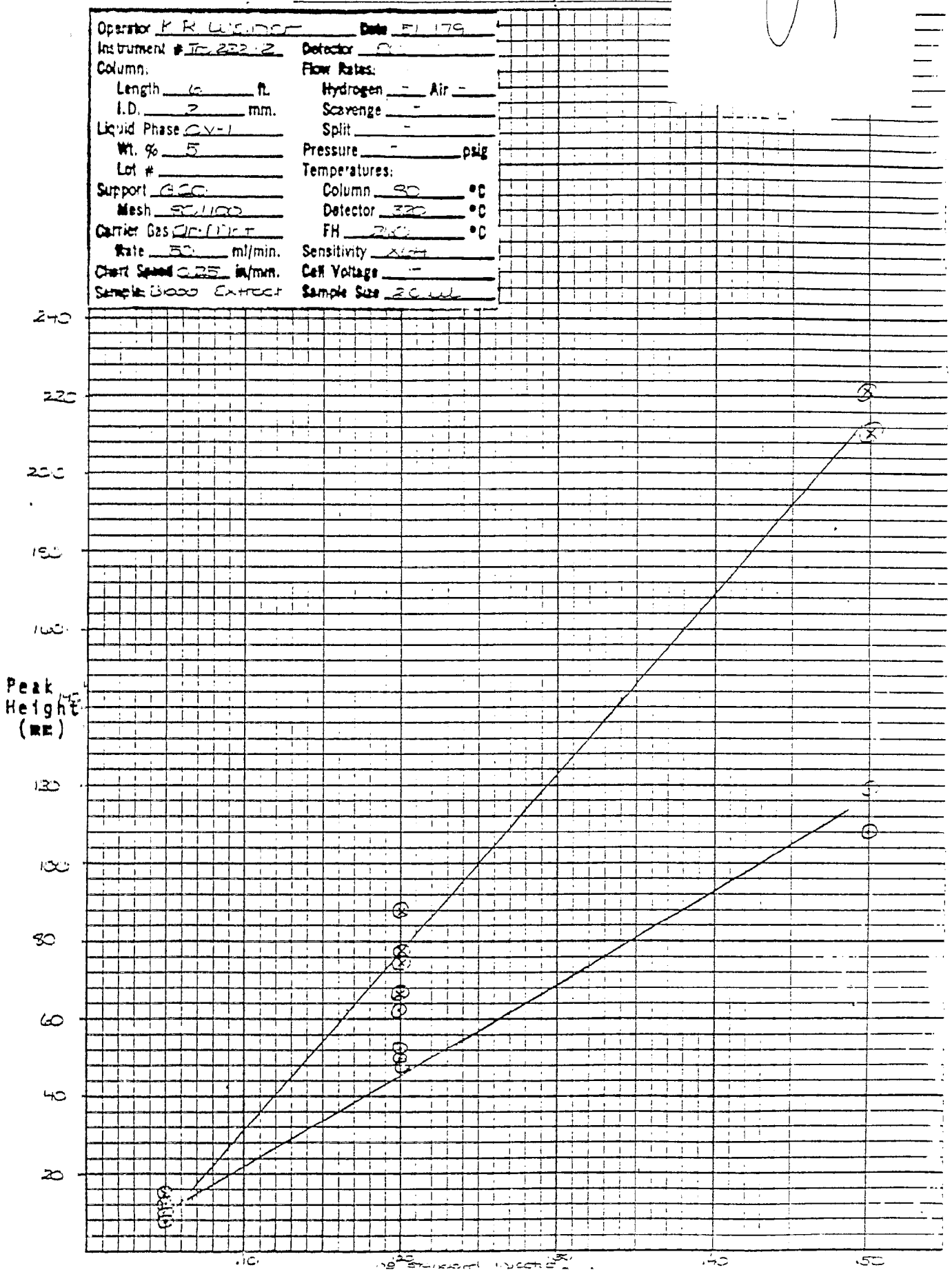


FIGURE 8. CATECHOL & HYDROQUINONE STANDARD CUR




46 0622

K+E 10 X 10 TO 1-1/4 INCH • 7 X 10 INCHES KEUFFEL & ESSER CO. MADE IN U.S.A.

DBZ0020390

79004A

FIGURE 9. TYPICAL CATECHOL AND HYDROQUINONE STANDARD CHROMATOGRAM




DBZ0020391

79004A

FIGURE 10. TYPICAL CHROMATOGRAM OF HYDROQUINONE & CATECHOL IN RAT BLOOD EXTRACT

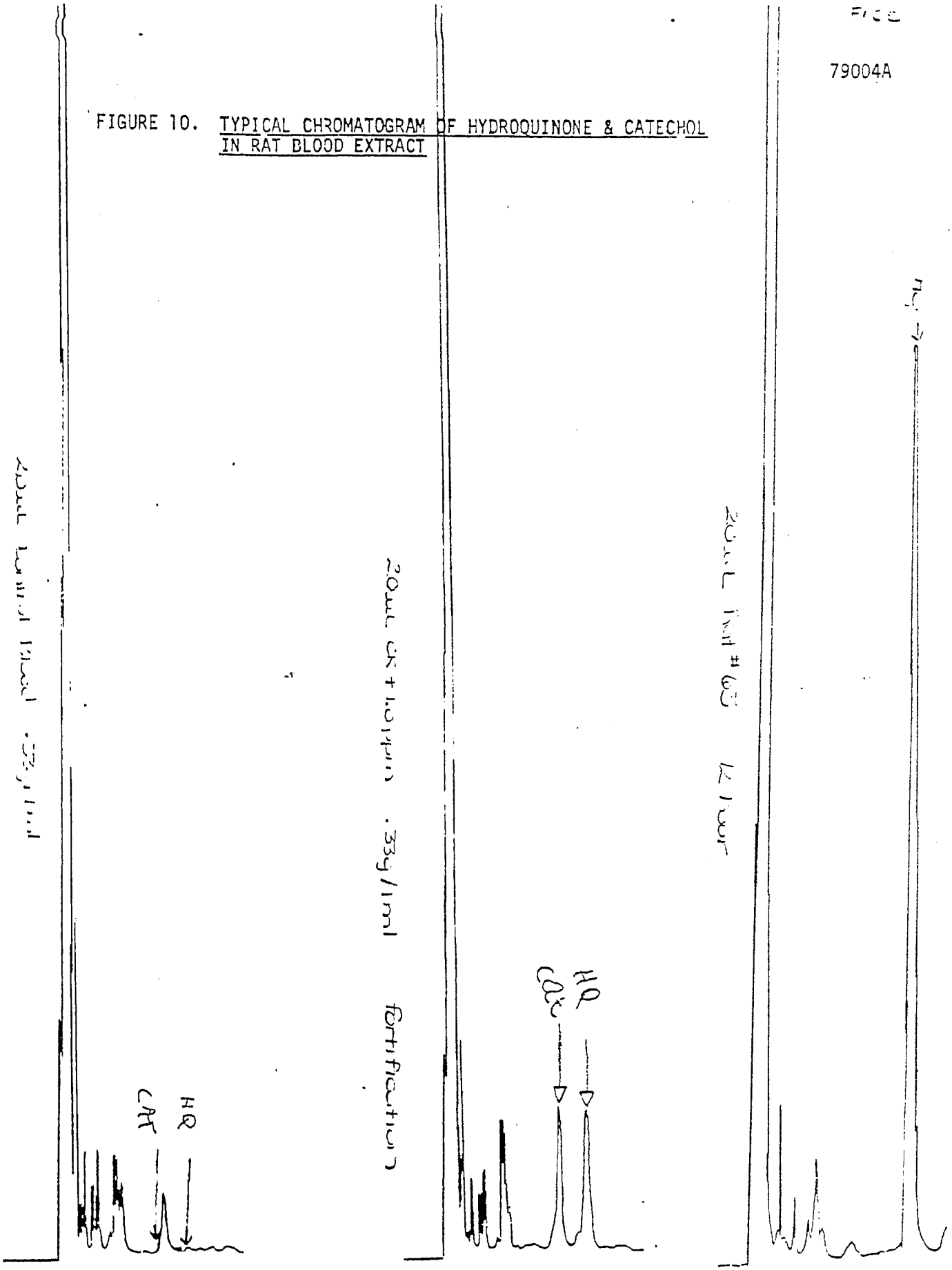
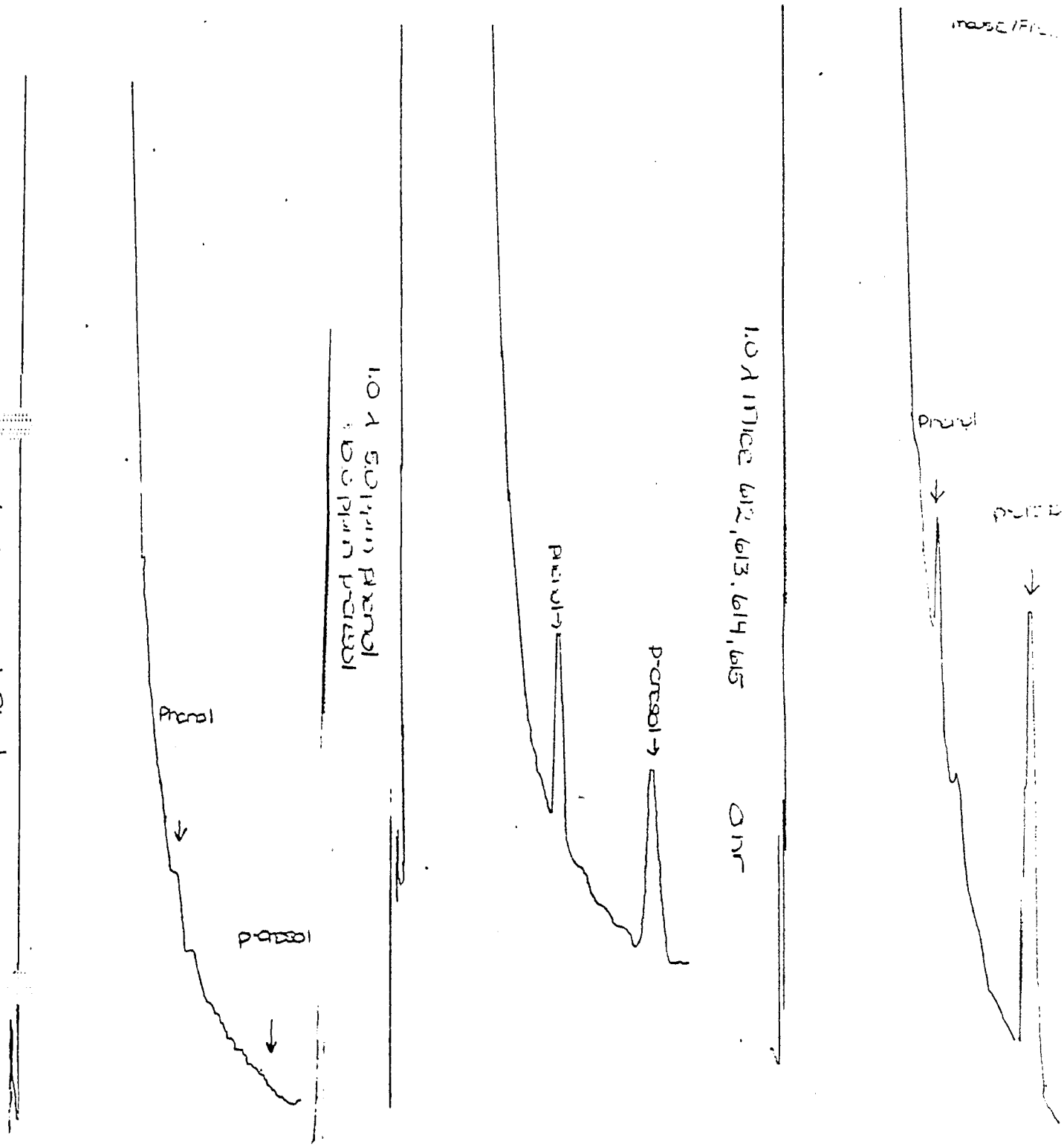


FIGURE 11. TYPICAL CHROMATOGRAM OF PHENOL IN MOUSE BLOOD EXTRACT



79004A

FIGURE 12. TYPICAL CHROMATOGRAM OF CATECHOL & HYDROQUINONE IN MOUSE BLOOD EXTRACT

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79004A

FIGURE 13. TYPICAL CHROMATOGRAM OF PHENOL IN INCUBATED RAT BLOOD EXTRACT

Catechol / Hydroquinone Rat

79004A

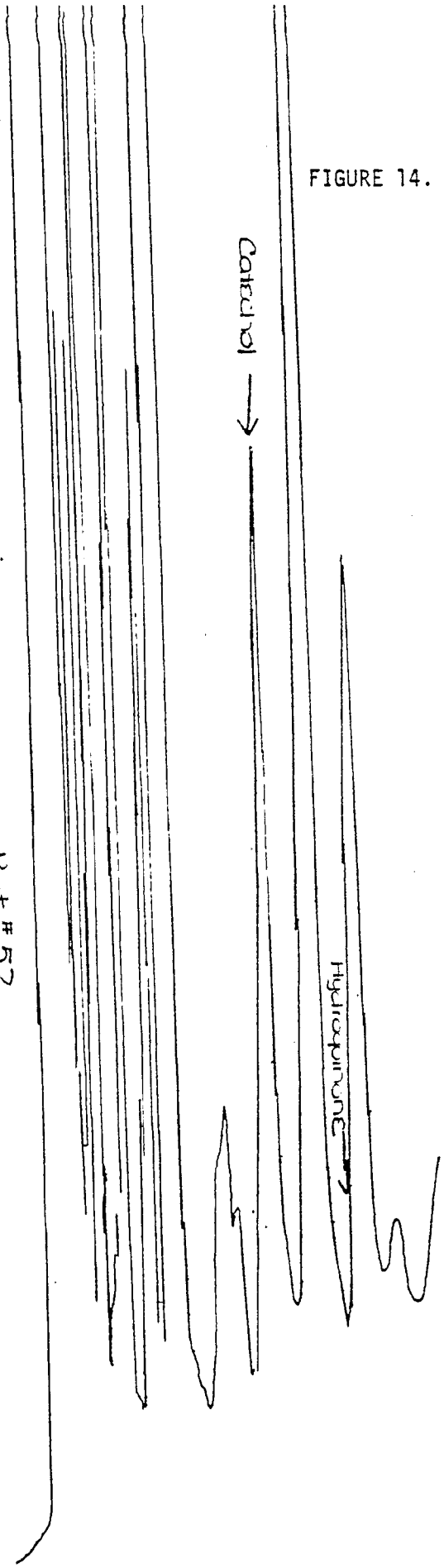


FIGURE 14. TYPICAL CHROMATOGRAM OF CATECHOL AND HYDROQUINONE IN INCUBATED RAT BLOOD EXTRACT

79004A

FIGURE 15. TYPICAL CHROMATOGRAM OF PHENOL IN INCUBATED MOUSE BLOOD EXTRACT

79004A

FIGURE 16. TYPICAL CHROMATOGRAM OF CATECHOL AND HYDROQUINONE IN INCUBATED MOUSE BLOOD EXTRACT




DBZ0020398