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ANALYSIS OF SOLUBLE BERYLLIUM BY GAS CHROMATOGRAPHY

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13. ABSTRACT

A new microanalytical method for the detection and quantitation of beryllium in aqueous samples and biological specimens has been developed. The method, based upon quantitation of chelated beryllium by gas chromatography, was employed to detect beryllium in both in vitro samples and tissues of rats given beryllium by injection. Beryllium was administered in the form of aqueous solutions of beryllium sulfate, and as little as 10 nanograms beryllium per gram of whole blood were detected and quantitated. In vivo studies which compared gas chromatographic analyses with radiometric analyses confirmed the validity of the new microanalytical technique. Preliminary studies were conducted to determine the ability of fluorinated chelating agents to remove beryllium oxide from the lungs of rats.

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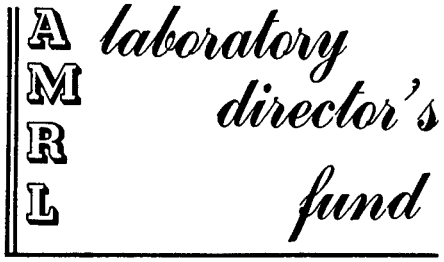
FOREWORD

This report was prepared by members of the Chemical Hazards Branch, Toxic Hazards Division of the Aerospace Medical Research Laboratory. The work was performed in support of Project 6302 "Toxic Hazards of Propellants and Materials," Task 630203 "Identification of Toxic Materials." Portions of the research reported were sponsored by the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio under contract no. F33615-71-C-1008 with Monsanto Research Corporation, Dayton, Ohio, Dr. W. G. Scribner, Principal Investigator. These contractual studies were supported by FY 1970 AMRL Laboratory Director's Fund. Other portions of the research were performed at Monsanto Research Corporation, Dayton, Ohio under Aerospace Research Laboratories contract no. F33615-69-C-1062, Dr. R. E. Sievers, Project Engineer. This report covers work conducted from July 1968 to November 1970.



This technical report has been reviewed and is approved.

ANTHONY A. THOMAS, MD
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Toxic Hazards Division
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INTRODUCTION

The toxicity of certain beryllium compounds has been recognized since the 1940s and the literature contains numerous publications which summarize aspects of beryllium toxicity (1, 2). However, the mechanism of toxicity of beryllium compounds has continued to elude researchers in this field. Since minute amounts of beryllium have apparently caused beryllium disease, biological research aimed at defining the mechanism of beryllium toxicity must use small doses of beryllium compounds to perform dose-response investigations, distribution studies, and eventual study of the effectiveness of therapeutics in removing beryllium from the animal body. Likewise, in industrial hygiene, bioenvironmental engineers must be able to determine beryllium in environmental samples and evaluate environmental conditions to ensure safety of workers. Public health officials must be prepared to assess the environmental beryllium levels to determine if a hazardous situation exists for the general public. Clearly, none of these various scientists can fulfill their tasks if analytical methods for determining submicrogram amounts of beryllium are lacking. Unfortunately, the methods that have been employed for detecting beryllium in biological material have neither the sensitivity nor specificity required and are capable of detecting minute amounts of beryllium only after extensive preparatory procedures requiring large samples. Thus, the techniques currently in widespread use, emission spectrography and Morin spectrophotofluorometry, have limitations especially when analyzing biological materials where only small sample sizes are available. In our laboratory we endeavored to develop a rapid, simple and yet ultransensitive analytical technique for determining beryllium in biological materials.

Inhouse and contractual research on the development of a gas chromatographic method for the detection of trace and ultratrace levels of beryllium in biological materials and aqueous samples is summarized in this report. This research was begun in 1967 and continues at the present time. The research consists of (1) the analysis of biological samples and aqueous solutions containing known beryllium concentrations, and (2) in vivo studies in which the beryllium content of organs and tissues from animals exposed to either beryllium sulfate or beryllium oxide were determined. The in vitro and in vivo studies have somewhat paralleled each other and, therefore, this report discusses the various experiments in the order in which they were conducted.

SECTION II

INITIAL IN VITRO STUDIES

Efforts to develop a gas chromatographic method for the detection and quantitation of beryllium in biological materials were initiated as a result of research reported in the literature (ref. 3) that suggested that ultratrace analysis of beryllium contained in such complex materials as blood and urine could quite likely be achieved. Our initial efforts to formulate a rapid, simple, yet ultrasensitive technique for quantitating beryllium in biological materials were successful and were published in the literature in 1968 (ref 4). In this early work it was demonstrated that as little as 5.95×10^{-6}gBe^{2+} per ml of blood could be accurately and precisely quantitated using a simple sealed tube reaction. In these studies, whole human blood, human urine, plant hemogenate, or monkey liver homogenate containing beryllium sulfate were combined with a benzene solution of trifluoroacetylacetone [H(tfa)], heated, treated to remove unreacted H(tfa), and then aliquots of the benzene layer injected directly into a chromatograph. Only 0.05 ml of blood and 0.1 ml of urine or 25% liver homogenate or plant extract were required in the analyses. Average time for the analyses was less than ½ hour.

Based upon this early success, the next questions to be answered were: (1) Is this simple technique capable of detecting and quantitating low levels of beryllium present in tissues of animals exposed to beryllium compounds? (2) Can the sensitivity of the gas chromatographic technique be improved to make it useful as a diagnostic tool in cases of human and animal exposure?

Early in our research we discerned that chomatographic efficiency must be optimized to accurately quantitate beryllium by this method. We found, for example, that the presence of metal surfaces and/or dead space in the pneumatic system must be avoided due to the adverse effects on both Be(tfa) and other compounds present in the solution being chromatographed. Thus, a glass column of minimal inside diameter and low dead volume at both the detector and injector ends is apparently essential for successful application of this technique. This opinion is given credence by the improvement in sensitivity that we obtained over our earlier work when a more desirable chromatographic system was employed. Choice of solid support is also of importance in the chromatography, since any trace of basic material in the chromatographic columns has an adverse effect on the results obtained.

The accuracy of our technique was enhanced by the addition of an internal standard to aid in quantitation and to eliminate volumetric errors inherent in the external standard method previously employed. We utilized a compound, symmetrical tetrabromoethane (TBE), which elutes in close proximity to beryllium trifluoroacetylacetonate and is ideal for use as an internal standard. The need for an internal standard was first realized when biological samples were analyzed. Sensitive settings and electron-capturing impurities gave rise to a broad tailing peak partially overlapping the beryllium peak. This is encountered more frequently when one is operating the chromatograph at sensitive settings, as we were, in an effort to detect low (<1 ppm Be^{2+}) levels of beryllium in biological materials. Under these conditions our concern was that the response obtained from a given amount of beryllium trifluoroacetylacetonate might be altered when varying amounts of interfering electron-capturing materials were encountered.

Initial work performed with the internal standard indicated that the chromatographic response obtained from a given quantity of TBE was approximately ⅛ that obtained from an equivalent quantity of beryllium. Thus, in our analytical standards, TBE was included in an amount equal to 8 times the beryllium present. This was done to obtain chromatographic peaks of about the same height at a given attenuation. Figure 1 illustrates the re-

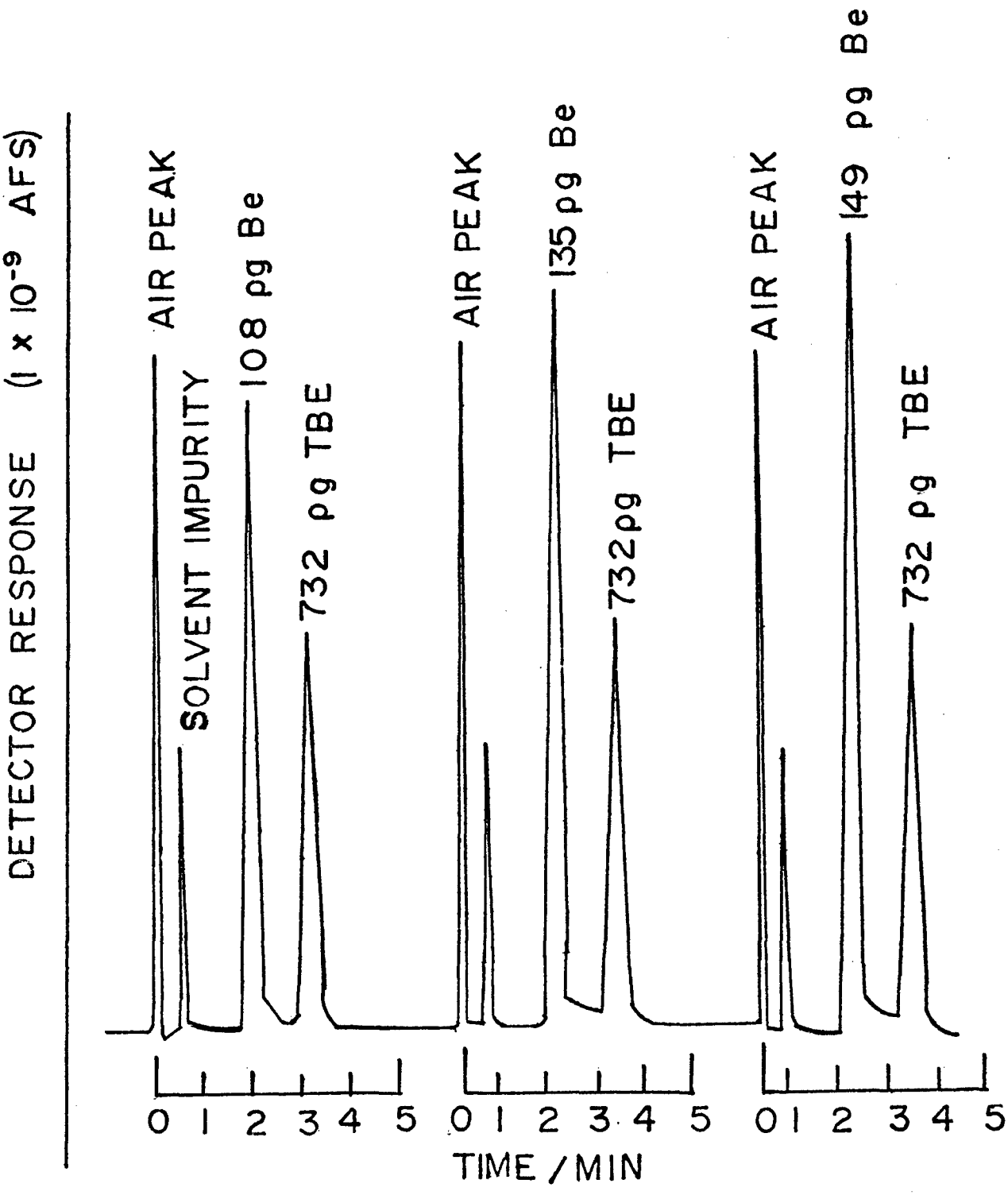

Figure 1. Chromatogram of Beryllium Trifluoroacetylacetonate and Sym-Tetrabromoethane. Beryllium was chromatographed as beryllium trifluoroacetylacetonate ($Be(tfa)_2$) but levels expressed are in terms of actual beryllium present.

sponse obtained from the injection of a working standard containing the amounts Be(tfa) as beryllium and TBE indicated. Initial interpretation of the chromatograms and preparation of standard curves proved to be a very complex task; however, a method was adopted which greatly facilitated calculations. The internal standard was included in the chelating solution [benzene solution of H(tfa)] so that each 0.5 ml of benzene contained not only trifluoroacetylacetone but also sym-tetrabromoethane. Calibration standards were prepared in which the concentrations of beryllium and tetrabromoethane were varied but the ratio between the two was always kept at 1:8. The instrument was calibrated by injecting the calibration standards, calculating the average ratio of the area of the TBE peak to the beryllium peak (area TBE/area Be) obtained from each standard, and then constructing a standard curve. The curve was drawn by first calculating two points, using a modification of a formula for internal standard use published by Schmit et al. (ref. 5). The formula developed is as follows:

$$\frac{C R_1 R_2}{K} = \text{Beryllium conc. in sample (nanograms/ml).}$$

where: C = Concentration of internal standard (TBE) in the chelating solution (nanograms/ml).

$$K = \frac{\text{Concentration of TBE}}{\text{Concentration of beryllium}} \text{ in standard solution}$$

$$R_1 = \frac{\text{Chromatogram peak area TBE}}{\text{Chromatogram peak area Be}} \text{ in calibration runs using standard solutions}$$

$$R_2 = \frac{\text{Chromatogram peak area Be}}{\text{Chromatogram peak area TBE}} \text{ in sample}$$

A sample calculation illustrating the use of the formula is:

$$C = 830 \text{ nanograms/ml TBE}$$

$$K = \frac{830 \text{ ng/ml TBE}}{103 \text{ ng/ml Be}} = 8.058$$

$$R_1 = \frac{3.46 \text{ cm}^2 = \text{area TBE peak}}{3.96 \text{ cm}^2 = \text{area Be peak}} = 0.875$$

$$R_2 = \frac{6.14 \text{ cm}^2 = \text{area Be peak}}{3.57 \text{ cm}^2 = \text{area TBE peak}} = 1.720$$

Therefore: Be conc. in sample $= \frac{CR_1R_2}{K} = \frac{830 \times 0.875 \times 1.720}{8.058} = 155 \text{ ng/ml}$

Implicit in use of the formula is the colinearity of the response of the chromatograph to $Be(tfa)_2$ and to TBE within a given concentration range. This is easily determined by injection of a series of standard solutions of TBE and beryllium. Tables I and II contain calibration data obtained by injection of standard solutions of TBE and $Be(tfa)_2$, and indicate the colinearity of response of various concentrations. These data are illustrated graphically in figure 2.

TABLE I

SYM-TETRABROMOETHANE CALIBRATION DATA

Standard g/ml	*Attenuation*	*Amount Injected (In pg)*	*Peak Dimension (In cm)*		*Peak Area (In cm^2)*
4.12×10^{-9}	2X	4.12	0.20 x 0.40		0.080
			0.21 x 0.45		0.095
			0.16 x 0.45		0.072
				Avg	0.082
4.12×10^{-8}	2X	20.6	0.20 x 1.30		0.26
			0.22 x 1.50		0.33
			0.20 x 1.25		0.25
			0.20 x 1.10		0.22
				Avg	0.27
4.12×10^{-8}	2X	41.2	0.20 x 2.55		0.51
			0.20 x 2.58		0.51
			0.20 x 2.50		0.50
			0.20 x 2.65		0.53
				Avg	0.51
4.12×10^{-7}	2X	123.6	0.20 x 6.72		1.34
			0.20 x 6.12		1.22
			0.20 x 6.08		1.22
			0.20 x 6.62		1.32
			0.20 x 6.55		1.31
				Avg	1.28
4.12×10^{-7}	2X	206	0.20 x 11.0		2.20
			0.20 x 10.6		2.12
			0.20 x 11.2		2.24
			0.20 x 11.5		2.30
			0.20 x 11.3		2.26
				Avg	2.22

TABLE I (Concluded)

SYM-TETRABROMOETHANE CALIBRATION DATA

Standard g/ml	*Attenuation*	*Amount Injected (In pg)*	*Peak Dimension (In cm)*		*Peak Area (In cm^2)*
4.12×10^{-7}	2X	329.6	0.20 x 18.5		3.70
			0.20 x 20.2		4.04
			0.20 x 19.9		3.98
			0.20 x 19.8		3.96
				Avg	3.92
4.12×10^{-7}	2X	412		Off Scale	
4.12×10^{-8}	8X	41.2	0.20 x 0.72		0.144
			0.20 x 0.72		0.144
			0.20 x 0.74		0.148
				Avg	0.145
4.12×10^{-7}	8X	206	0.20 x 3.08		0.616
			0.20 x 3.02		0.604
			0.20 x 3.08		0.616
				Avg	0.612
4.12×10^{-7}	8X	412	0.20 x 6.09		1.22
			0.20 x 6.00		1.20
			0.20 x 6.15		1.23
				Avg	1.22
4.12×10^{-6}	8X	2,060	0.30 x 16.05		4.82
			0.28 x 15.82		4.43
			0.28 x 15.92		4.46
				Avg	4.57

TABLE II

BERYLLIUM TRIFLUOROACETYLACETONATE CALIBRATION DATA

Solution g/ml	*Attenuation*	*Amount Injected (In pg)*	*Peak Dimension (In cm)*		*Peak Area (In cm^2)*
5.58×10^{-9}	2X	1.67	0.14 x 2.48		0.347
			0.13 x 2.51		0.326
			0.13 x 2.40		0.312
				Avg	0.328
5.58×10^{-9}	2X	2.29	0.12 x 4.38		0.526
			0.12 x 3.72		0.446
			0.12 x 4.20		0.504
				Avg	0.492
5.58×10^{-9}	2X	4.46	0.12 x 6.28		0.754
			0.14 x 6.25		0.875
			0.13 x 6.25		0.813
				Avg	0.814
5.58×10^{-9}	2X	5.58	0.13 x 7.17		0.932
			0.14 x 7.50		1.05
				Avg	0.949
5.58×10^{-8}	2X	16.7	0.13 x 15.8		2.05
			0.14 x 16.4		2.29
			0.14 x 16.3		2.28
				Avg	2.21
5.58×10^{-9}	8X	5.58	0.13 x 1.21		0.157
			0.14 x 1.26		0.176
			0.14 x 1.20		0.168
				Avg	0.167
5.58×10^{-8}	8X	16.7	0.13 x 3.08		0.400
			0.13 x 3.00		0.390
				Avg	0.395

TABLE II (Concluded)

BERYLLIUM TRIFLUOROACETYLACETONATE CALIBRATION DATA

Solution g/ml	*Attenuation*	*Amount Injected (In pg)*	*Peak Dimension (In cm)*		*Peak Area (In cm^2)*
5.58×10^{-8}	8X	27.9	0.15 x 4.50		0.675
			0.15 x 4.08		0.612
			0.14 x 4.69		0.657
				Avg	0.648
5.58×10^{-8}	8X	44.6	0.16 x 6.70		1.07
		8			
5.58×10^{-8}	8X	55.8	0.16 x 7.80		1.25
			0.16 x 8.02		1.28
			0.17 x 8.50		1.45
			0.17 x 8.25		1.40
				Avg	1.35

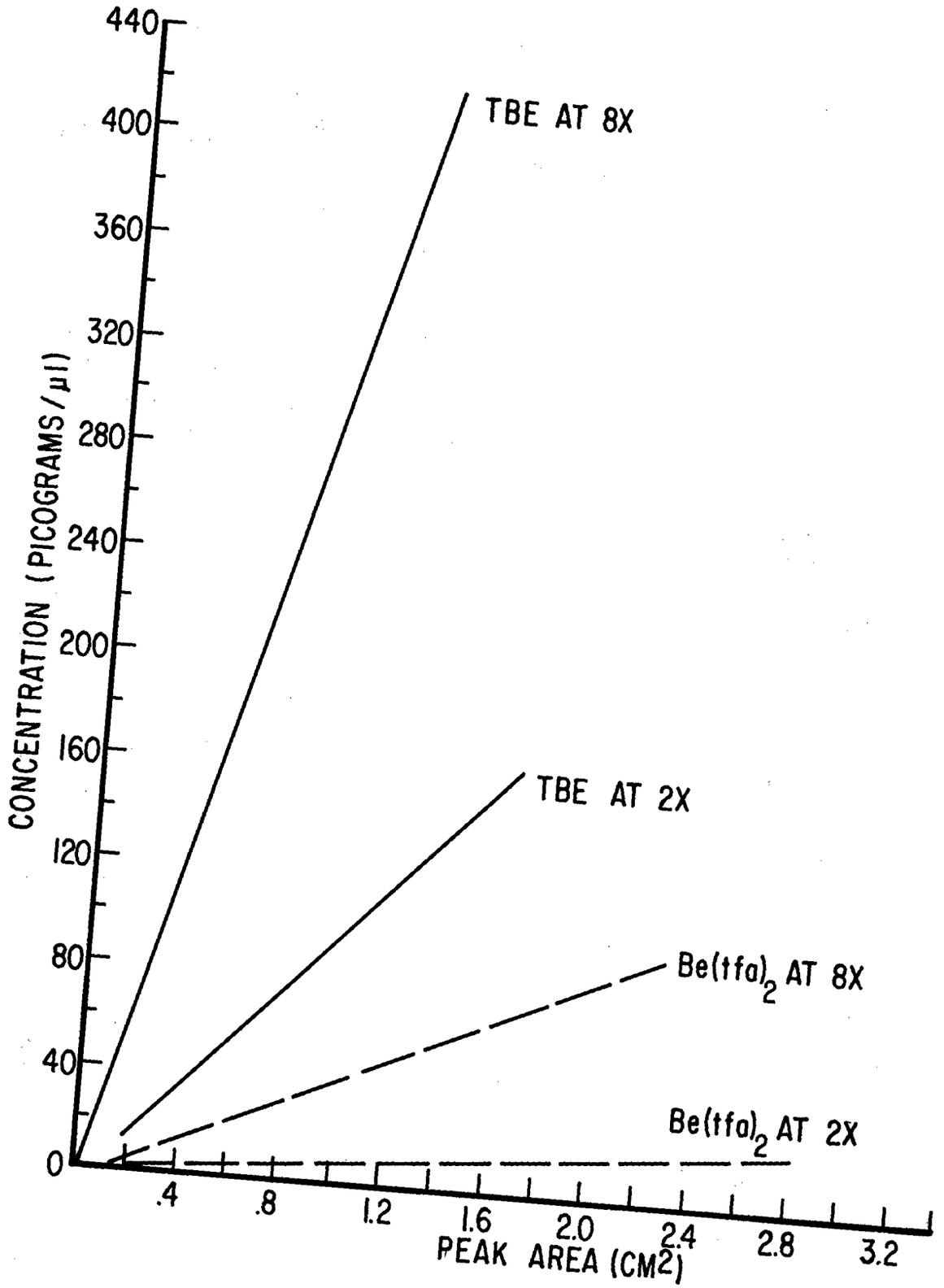

Figure 2. Calibration Plots for $Be(tfa)_2$ and TBE. These plots illustrate the linearity of response of the chromatograph over a wide range for both Be $(tfa)_2$ and TBE.

SECTION III

IN VITRO STUDIES TO OPTIMIZE PROCEDURES

A series of studies was undertaken to determine the optimum conditions for quantitative analysis of submicrogram amounts of ionic beryllium in water or in blood. Standard aqueous solutions were prepared, analyzed by gas chromatography, and the percent recovery calculated. Standard deviations were determined where appropriate. These aqueous beryllium standards were then combined with whole blood and the spiked blood samples analyzed.

A. Experimental Details

1. Materials & methods

Benzene: ACS quality, thiophene free, Matheson, Coleman and Bell, Norwood, Ohio.

Trifluoroacetylacetone: Pierce Chemical Co., Rockford, Illinois or Peninsular Chemresearch, Inc., Gainesville, Florida. Material was twice distilled before use. Fraction distilling at 105-106C was stored under nitrogen in a teflon bottle. Stored material is stable for 6 weeks.

Saturated Disodium EDTA Solution: 30.0 g of Na_2 EDTA, chemically pure, c.p., Cambridge Chemical Products, Ind., Dearborn, Michigan, was dissolved in double-distilled water of sufficient quantity to make 100 ml.

Sym-Tetrabromoethane (TBE): Eastman Kodak Company, Rochester, New York. Material distilling at 117C (15 mm Hg) was collected and used fresh. Distilled material can be sealed in glass ampules (under dry N_2) and stored for about 4 weeks.

Beryllium: 99% pure, 200 mesh, Alfa Inorganics, Inc., Beverly, Massachusetts.

Beryllium Trifluoroacetylacetonate ($Be(tfa)_2$): Synthetized from direct reaction of metallic beryllium and trifloroacetylacetone as reported previously (4). Purified by recrystallization and two sublimations (M.P. 112C). Compound can be retained in a sealed container (screw cap vial) for at least 8 months without degradation.

Ammonium Hydroxide: 28%, Analytical Reagent Grade, Mallinckrodt Chemical Works, St. Louis, Missouri.

Sulfuric Acid: ACS Quality, Fisher Chemical Company.

Water: Double distilled (glass still).

TBE Stock Solutions: A benzene solution of TBE was prepared by dissolving 0.5270 g of TBE in sufficient benzene to make 250 ml (TBE conc. = 2.108×10^{-3} g/ml). A working stock solution was prepared by diluting 0.1 ml of the stock solution to 25 ml (TBE conc. = 8.432×10^{-6} g/ml).

Beryllium Trifluoroacetylacetonate ($Be(tfa)_2$) stock solution: Beryllium trifluoroacetylacetonate, 0.0431 g was placed in a 100 ml volumetric flask and benzene was added to make 100 ml. (Be conc = 12.33×10^{-6} g Be/ml).

Chelating Solutions: Two chelating solutions were prepared. For the analysis of samples having a beryllium concentration of 1.14×10^{-6} g/ml, the solution was prepared to contain 8.43×10^{-6} g TBE internal standard/ml and 4 μl H(tfa)/ml. For the analysis of samples having a lower beryllium concentration, a chelating solution containing 8.43×10^{-6} g TBE and 1 μl H(tfa)/ml was used.

Calibration Standards: The $Be(tfa)_2$ stock solution was diluted 1:10 to make a solution containing 1.23×10^{-6} g Be/ml. Aliquots of this solution (containing 8.43×10^{-6} g TBE/ml) were placed in graduated centrifuge tubes and sufficient benzene added to make 10 ml. The concentration of beryllium in each tube ranged from 0.062×10^{-7} g Be/ml to 1.230×10^{-7} g Be per ml. The TBE concentration in each tube was either 8.43×10^{-7} g TBE/ml or 8.43×10^{-6} g TBE/ml.

2. Instrumentation

Instrument: A Varian 2100 Gas Chromatograph equipped with electron capture detector (250 millicuries Tritium, D.C. mode) was used for the chromatography. Temperatures—injector, 140C, detector, 180C, column, 110C. Carrier gas (Prepurified Nitrogen, J. T. Baker Chemical Company, East Chicago, Indiana) flow = 100 cc/min. Chromatography was effected on 6' x 2 mm i.d. glass u-tubes packed with 10% SE-52 silicone gum on Gas Chrom Z (Applied Science Lab., State College, Pennsylvania). On this column, retention indices for beryllium trifluoroacetylacetonate and tetrabromoethane are 12.00, 12.80 methylene units (7) respectively.

B. Analysis of Aqueous Standards

Add 0.05 ml of sample to an ampule plus 0.5 ml of chelation solution. Seal ampule. Allow to cool, then shake 20 seconds. Heat @ 115C for 15 minutes and open ampule after cooling. Add 80 μl of 28% NH_4OH, stopper ampule and shake for 10 seconds. Centrifuge 10 minutes, analyze 1 μl aliquots of the benzene layer.

In two series of experiments, aqueous solutions ranging in concentration from 0.002×10^{-6} g Be/ml to 1.14×10^{-6} g Be/ml were analyzed. Table III gives the results obtained in the first series.

TABLE III

ANALYSIS OF 0.228 TO 1.14 PPM AQUEOUS BERYLLIUM SOLUTIONS

Be^{2+} Conc Prepared (g/ml)	*Be^{2+} Found (g/ml)*	*Avg % Recov*
1.14×10^{-6}	0.96×10^{-6}	84
0.912×10^{-6}	0.93×10^{-6}	101
0.684×10^{-6}	0.61×10^{-6}	89
0.456×10^{-6}	0.35×10^{-6}	76
0.228×10^{-6}	0.20×10^{-6}	86

In the second series, aqueous solutions containing 0.0912 to 0.002×10^{-6} g Be/ml were analyzed; the results are listed in table IV.

TABLE IV

ANALYSIS OF 0.002 TO 0.0912 PPM AQUEOUS BERYLLIUM SOLUTIONS

Be^{2+} Conc Prepared (g/ml)	*Be^{2+} Found (g/ml)*	*Avg % Recov*
0.0912×10^{-6}	0.069×10^{-6}	75.3
0.0684×10^{-6}	0.045×10^{-6}	65.5
0.0456×10^{-6}	0.025×10^{-6}	54.2
0.0228×10^{-6}	0.010×10^{-6}	43.9
0.002×10^{-6}	0	0
Distilled Water	0	0

C. Analysis of Spiked Blood

The aqueous beryllium stock solution used in the previous section was used to prepare heparinized human blood samples containing levels of beryllium identical to those aqueous solutions as described in the previous section. Two series of spike blood samples were prepared and analyzed.

Aliquots of a beryllium stock solution (1.14×10^{-5} g Be^{2+}/ml) ranging from 20 μl to 100 μl were combined with sufficient amounts of heparinized whole human blood to make 1.0 ml samples. Five samples were prepared ranging in concentration from 0.228×10^{-6} Be/ml blood to 1.14×10^{-6} g Be/ml. Similarly, a second series of spiked samples was prepared by combining aliquots of an aqueous beryllium standard containing 0.114×10^{-5} g Be^{2+}/ml with heparinized human blood to make 1.0 ml spiked samples ranging in concentration from 0.0228×10^{-6} g Be/ml to 0.114×10^{-6} Be/ml.

The spiked blood samples were analyzed in the same manner as reported for the aqueous samples except that less ammonia is required to remove unreacted H(tfa) when blood is present in the reaction mixture. In the analysis of spiked blood containing greater than 0.114×10^{-6} g Be/ml, 50 μl of 28% NH_4OH was found to be the optimal amount. In the analysis of spiked blood containing less than 0.114×10^{-6} g Be^{2+}/ml, 40 μl of 28% NH_4OH was found to be optimum. Analytical results are shown in table V.

D. Discussion

These studies indicate that analysis of aqueous solutions as well as spiked blood samples containing as little as 0.027×10^{-6} g Be/ml can be performed accurately and precisely using gas chromatography. Also apparent from the results is the fact that the gas chromatographic analysis of spiked blood resulted in better results (higher % recoveries) than the analysis of aqueous beryllium solutions containing the same amount of beryllium.

In Table V a comparison is made between two methods of calculating the analytical results. Beryllium was determined using both the internal standard method and the more simple beryllium peak area measurement. The use of the internal standard appears to correct for the apparent losses of $Be(tfa)_2$. Due to the improved recoveries, the use of the internal standard is recommended.

TABLE V

ANALYSIS OF BLOOD CONTAINING 0.023 TO 1.14 PPM BERYLLIUM

Gms/ml Be^{2+} Added to Blood	*Gms/ml Found (% Recovery, # Determ.)*	
	Internal Standard	*Be Peak Area*
1.140×10^{-6}	1.050×10^{-6} (92%, 6)*	0.913×10^{-6} (80%, 6)**
0.912×10^{-6}	0.850×10^{-6} (93%, 5)	0.610×10^{-6} (66.5%, 5)
0.684×10^{-6}	0.580×10^{-6} (85%, 4)	0.410×10^{-6} (60.4%, 4)
0.456×10^{-6}	0.480×10^{-6} (105%, 6)	0.313×10^{-6} (68.6%, 6)
0.228×10^{-6}	0.243×10^{-6} (107%, 6)	0.123×10^{-6} (54%, 6)
0.114×10^{-6}	0.110×10^{-6} (100%, 4)	0.097×10^{-6} (85%, 4)
0.091×10^{-6}	0.080×10^{-6} (88%, 3)	0.064×10^{-6} (71%, 3)
0.068×10^{-6}	0.064×10^{-6} (94%, 3)	0.053×10^{-6} (78%, 3)
0.046×10^{-6}	0.036×10^{-6} (78%, 2)	0.030×10^{-6} (65%, 2)
0.023×10^{-6}	0.027×10^{-6} (117%, 2)	0.022×10^{-6} (97%, 2)

*Standard deviation = $\pm 0.075 \times 10^{-6}$
**Standard deviation = $\pm 0.15 \times 10^{-6}$

SECTION IV

IN VIVO STUDIES USING BERYLLIUM SULFATE

A. Gas Chromatographic Studies

This study was undertaken to determine if beryllium could be detected and quantitated in tissues from animals administered beryllium sulfate. Portions of the results of these experiments were reported at the 5th Annual Conference on Atmospheric Contamination in Confined Spaces, Dayton, Ohio (ref. 6).

White, male laboratory rats (Charles River Laboratories) weighing 350-400 gm were employed in these experiments and were provided with food and water ad libidum. The rats were anesthetized with ether and given 2.3 mg ionic beryllium per kg body weight intravenously (tail vein). One hour after injection the rats were reanesthetized and about 1 milliliter of blood was collected* in either a plastic or silanized glass vial containing 2 drops of Na-heparin solution. The rats were bled in the same manner at 3 hours post-injection. At 24 hours postinjection the animals were bled as before and, while under ether anesthesia, the spleen and a portion of the liver were excised. Homogenates of the harvested organs were prepared by grinding 200-300 mg of tissue with 0.2-0.03 ml of 1M Na acetate. Fifty μl of these homogenates were analyzed using the same procedures as blood. Values given in table VI are the averages of two or three analyses performed on each blood or tissue sample.

*from the capillary bed behind the eye

TABLE VI

MICROGRAMS BERYLLIUM PER ML BLOOD OR PER GRAM TISSUE FOLLOWING I.V. ADMINISTRATION OF 2.3 MG BERYLLIUM PER KG

Rat #	*1 Hour Blood*	*3 Hour Blood*	*24 Hour Blood*	*Liver*	*Spleen*
1	2.4	2.2	0.05	0.80	2.1
2	2.0	1.5	ND	0.45	1.3
3	1.5	1.0	0.05	ND	1.5
4	2.5	1.6	0.05	0.21	0.86
5	5.1	4.2	0.03	0.10	5.2
6	2.4	2.4	0.10	0.05	2.7
7	1.9	1.3	0.06	0.10	2.4
8	2.7	1.6	0.05	0.10	1.9
9	2.4	1.6	0.07	0.09	2.3
10	2.6	1.8	0.05	0.11	1.8
Controls (5)	ND	ND	—	—	ND

ND = None detected

In a second experiment, male rats weighing 450-600 g were injected with 5 mg ionic beryllium per kilogram of body weight. Animals were bled ½ hour postinjection and again at 24 hours postinjection at which time liver samples and spleens were taken for analysis. The analyses of these tissues gave the results shown in table VII.

TABLE VII

MICROGRAMS BERYLLIUM PER ML BLOOD OR PER GRAM TISSUE FOLLOWING I.V. ADMINISTRATION OF 5 MG BERYLLIUM PER KG

Rat #	*½ Hour Blood*	*24 Hour Blood*	*Liver*	*Spleen*
1	2.9	0.68	5.2	2.3
2	2.8	0.80	4.1	*
3 (Control)	ND	ND	ND	ND
4	1.3	*	0.94	*
5	3.8	0.35	3.8	3.4
6	1.6	*	1.1	1.0
7 (Control)	ND	ND	ND	ND

ND = None detected
* = Samples deteriorated prior to analysis.

A third study was performed in this series, to determine if the gas chromatographic technique could be used to detect and quantitate extremely low levels of beryllium in vivo samples. Two rats were given 0.5 mg Be^{2+}/kg via tail vein injection. These rats as well as one untreated control were bled from the capillary bed behind the eye at one hour and three hours post treatment. At three hours the animals were sacrificed and livers and spleens harvested. Results are given in table VIII.

TABLE VIII

MICROGRAMS BERYLLIUM PER ML BLOOD OR PER GRAM TISSUE FOLLOWING I.V. ADMINISTRATION OF 0.5 MG BERYLLIUM PER KG

Rat #	*1 Hour Blood*	*3 Hour Blood*	*Liver**	*Spleen**
1	0.36 (4)	0.04 (2)	0.21 (4)	0.13 (2)
2	0.078 (5)	ND (4)	0.18 (2)	0.12 (3)
Control	ND (2)	ND (2)	ND (2)	ND (2)

ND = None detected
* = animals sacrificed at 3 hours postinjection

Number of determinations is shown in parenthesis

Standard deviations:

Rat #1 — 1-hr blood = $\pm$ 0.040 x 10^{-6}
Rat #1 — liver = $\pm$ 0.047 x 10^{-6}
Rat #2 — 1-hr blood = $\pm$ 0.036 x 10^{-6}

These results clearly illustrate the efficacy of the chelation — gas chromatographic technique for determining at least one form of beryllium, ionic beryllium, in tissues of treated animals.

B. Radiochemical Studies

Findings in the studies employing non-radioactive beryllium sulfate injected I.V. were in some ways puzzling. Levels of beryllium determined by gas chromatographic analysis were found to be rather independent of the actual dose given and, if anything, indicated an inverse relationship. That is, in some animals given only 0.5 mg Be^{2+}/kg, the beryllium levels found were not markedly different from beryllium levels observed in the tissues of animals administered 5 to 10 times more beryllium. These findings raised the question that perhaps the animal was in some way metabolizing a certain portion of the administered beryllium and was converting the metal to some bound form which was insensitive to chelation, extraction, and subsequent analysis. To verify the obversed distribution phenomena, we formulated a study in which ^{7}Be labelled beryllium surfate solutions were injected into rats and the beryllium in various harvested tissues was assayed radiochemically. This portion of the research was performed at Monsanto Research Corporation Laboratories under Aerospace Research Laboratories Contract #F33615-69-C-1062. Dr. R. E. Sievers was the Project Engineer.

A solution of beryllium was prepared by placing 58.7 mg of powdered beryllium metal (200 mesh, Alfa Inorganics, Beverly, Mass) in a 25 ml volumetric flask, adding about 8 ml of distilled water and sufficient concentrated sulfuric acid (20-30 drops) to dissolve the powdered metal. Radioactive beryllium (^{7}Be, 160 μc, New England Nuclear Corporation) was added followed by distilled water to make 25 ml. The resulting solution (pH 1.8) contained 2.35 mg Be/ml and 6.8 μc ^{7}Be/ml. A second solution was prepared by diluting an aliquot of the first solution 1:5 and adding sufficient ^{7}Be to make 17 μc ^{7}Be/ml, resulting in a Be^{2+} concentration of 0.47 mg Be/ml at pH 2.4.

Three groups of white rats weighing 160-200 g were administered labelled beryllium sulfate solutions I.V. (tail vein) while under ether anesthesia. Doses administered were corrected for syringe dead volume and represent the actual amount of beryllium administered. These doses were 0.65 mg/kg (Series I), 3.4 mg/kg (Series II), and 5.3 mg/kg (Series III). Animals were bled (eye method) at 1, 3 and 24 hours postinjection. At 24 hours the animals were sacrificed and the livers and spleens harvested for analysis.

All blood and tissue samples were placed in individual counting vials and counted for two 5-minute intervals. Counts obtained were corrected for background and then multiplied by a factor (determined daily using weighed aliquots of standard solutions) to arrive at the amount of beryllium in milligrams. This manipulation was done using a single computer program.

Results are tabulated in table IX. These data are similar in many ways to the results obtained in the gas chromatographic studies. In terms of animal to animal differences within a given group of rats, as well as the lack of close agreement between dose administered and amount of beryllium determined and the order of magnitude of beryllium found in the various organs and tissues, these radiochemical data compare very favorably with the gas chromatographic data reported in the previous section.

These results provided much substantiation for our gas chromatographic findings; however, since duplicate analyses employing the radiochemical method and the gas chromatographic method were not run on the same sample, only a qualitative comparison of the two analytical methods could be made. The following section describes in vivo work intended to demonstrate unequivocally the accuracy and precision of the gas chromatographic method analysis.

TABLE IX

RADIOCHEMICAL ANALYSES OF RAT TISSUES AFTER I.V. ADMINISTRATION OF BERYLLIUM

Sample	*Rat #1*	*Rat #2*	*Rat #3*	*Rat #4*	*Rat #5*	*Rat #6*
			Series I (0.65 mg Be/kg)			
Blood 1 hr	0.19	0.86	0.35	0.18	0.23	—
Blood 3 hr	0.09	0.45	0.13	0.08	0.08	—
Blood 24 hr	0.02	0.02	0.02	0.01	0.01	—
Liver	0.006	0.009	0.009	0.006	0.005	—
Spleen	0.008	0.01	0.02	0.008	0.009	—
			Series II (3.4 mg Be/kg)			
Blood 1 hr	8.2	6.0	2.8	1.6	7.0	—
Blood 3 hr	2.9	3.2	0.64	0.76	2.1	—
Blood 24 hr	0.17	0.16	0.15	0.09	0.13	—
Liver	3.2	1.3	3.3	0.03	1.8	—
Spleen	3.9	0.83	32.0	0.07	18	—
			Series III (5.3 mg Be/kg)			
Blood 1 hr	3.2	2.3	2.7	3.5	3.7	0.95
Blood 3 hr	0.84	1.4	1.4	2.3	0.88	—
Blood 24 hr	—	0.22	0.16	0.18	—	—
Liver	11	0.09	0.11	0.13	8.1	0.19
Spleen	83	0.13	0.07	0.09	51	0.29

Values given in above table are in μg Be/ml blood or μg Be/ tissue.

SECTION V

COMBINED G. C. & R. C. STUDIES FOR COMPARATIVE PURPOSES

Three groups of four rats were given doses of beryllium sulfate (prepared and administered in the same manner as outlined previously) while the animals were under ether anesthesia. Appropriate amounts of solution were given so that Series I rats received 5.6 mg/kg, Series II received 2.5 mg/kg and Series III received 0.53 mg/kg. All doses are corrected for syringe dead volume and represent the actual amount of beryllium which was injected into the animal. At 1 and 4 hours postinjection the rats were reanesthetized and about 0.5-1.5 ml blood (obtained using the eye method) was placed in a tared counting vial containing 0.2 ml of Na_2EDTA solution. At 4 hours the animals were sacrificed and a portion of the liver plus the whole spleen excised. Tissue samples were placed into tared counting vials. Blood and tissue vials were weighed, counted twice (5 min counts) and the beryllium present calculated after corrections for background and radioactive decay were applied.

These blood and tissue samples were then analyzed by gas chromatography in a single blind experiment. Details of the method are the same as outlined previously with the following exception:

A 25% tissue homogenate was prepared by homogenizing 500 mg of tissue with 1.3 ml of 1M sodium acetate and 0.2 ml of a saturated solution of disodium EDTA (Camco Chemical Company). No changes in instrument conditions were made. The internal standard was employed in a slightly different manner than before.

Two stock solutions were prepared. Sym-tetrabromoethane, 5.540 mg, was placed in a 100 ml volumetric flask and benzene added to make 100 ml. Beryllium trifluoroacetylacetonate, 7.088 mg, was placed in a 250 ml volumetric flask and benzene added to make 250 ml (Be concentration = 8.108×10^{-7} g/ml). Working standards were prepared by taking 0.16 ml of the TBE stock + 0.1 to 2.0 ml volumes of the beryllium stock and adding benzene to make 10 ml. The chelating solution was formulated to contain 4 μl H(tfa) per ml and the same concentration of TBE as in the working standards (8.86×10^{-7} g/ml). The chromatograph was calibrated daily by injecting the working standards and plotting the ratios (Be peak area/TBE peak area) on the ordinate versus beryllium concentration on the abscissa. Beryllium concentrations in the unknowns were determined by calculating the ratio of the area of the beryllium peak to the area of the TBE peak and reading the corresponding beryllium concentration from the standard curve. Three to six replicate analyses were performed on each blood or tissue sample and these results were averaged. The data obtained with the two analytical procedures are compared in table X. In Series III, the procedure for analyzing tissue had been completely optimized and thus these results are the best. In addition to the use of 1M sodium acetate and saturated disodium EDTA as a homogenizing medium, we also determined that the tissue must be *very* thoroughly homogenized for at least 5 minutes to obtain acceptable recoveries.

These results demonstrate unequivocally the efficacy of the gas chromatographic method for determining beryllium in the blood and tissues of rats given I.V. doses of beryllium sulfate. The lower limit of detection and quantitation is shown to be less than 0.04 μg Be/g blood.

TABLE X

COMPARISON OF DATA OBTAINED BY GAS CHROMATOGRAPHIC AND RADIOCHEMICAL ANALYSES OF BLOOD AND TISSUE OF RATS ADMINISTERED BERYLLIUM I. V.

	Blood											
	1 Hour			*4 Hours*			*Liver*			*Spleen*		
Rat	*R**	*G***	*%****	*R*	*G*	*%*	*R**	*G***	*%****	*R*	*G*	*%*
	Series I - 5.6 mg/kg						*Series I - 5.6 mg/kg*					
1	1.72	1.41	82	0.53	0.47	89	1.53	0.88	58	0.97	0.71	73
2	7.20	6.9	96	4.36	3.7	85	4.22	1.63	39	1.60	1.34	84
3[1]	—	—	—	—	—	—	—	—	—	—	—	—
4	0.60	0.48	80	0.13	0.06	46	0.79	0.27	34	0.44	0.4	91
	Series II - 2.5 mg/kg						*Series II - 2.5 mg/kg*					
1	3.21	2.7	84	1.33	1.4	105	2.44	1.22	50	1.68	0.88	52
2	0.28	0.27	96	0.044	0.029	66	0.57	0.24	42	0.18	0.11	61
3	0.38	0.38	100	0.059	0.051	86	0.67	0.55	82	0.24	0.12	50
4	2.34	2.2	94	1.03	0.95	92	1.82	1.3	71	1.13	0.69	61
	Series III - 0.53 mg/kg						*Series III - 0.53 mg/kg*					
1	2.16	2.62	121	0.99	1.09	110	1.73	1.84	106	1.00	0.61	61
2	3.02	3.37	112	1.68	1.73	103	1.86	1.37	74	1.27	1.11	87
3	5.88	5.79	98	3.95	4.56	115	3.04	2.76	91	1.55	1.81	117
4	0.076	0.08	105	0.010	0.012	120	0.33	0.36	109	0.077	0.13	169

*R = Radiotracer Method
**G = Gas Chromatography
***% = $\frac{G}{R} \times 100$
[1] = Beryllium administration resulted in tissue perforation
Values in the tables are μg Be/ml blood or μg Be/g tissue

SECTION VI

EFFECTS OF B-DIKETONE VAPORS IN INTRATRACHEALLY ADMINISTERED BERYLLIUM OXIDE

This work performed in conjunction with Monsanto Research Corporation under contract to Aerospace Research Laboratories involved the synthesis of radioactively labelled beryllium oxide and the intratracheal administration of this compound in the lungs of white rats. These animals were then treated with vapors of trifluoroacetylacetone and hexafluoroacetylacetone to determine if any of the beryllium oxide was removed from the rat lungs during inhalation of the fluorinated chelating agents. Labelled BeO was prepared as follows:

Beryllium nitrate, $Be(NO_3)_2 \cdot 3H_20$, (7.5 g) was dissolved in 70 ml of water and 32 μc of ^7Be (2.0 ml of 16 μc Be/ml as $^7BeCl_2$) was added. The pH was adjusted to about 11 by the addition of 10N sodium hydroxide and quantitative precipitation of beryllium (as beryllium hydroxide) was obtained (supernatant contained no radioactivity). The beryllium hydroxide was filtered, dried, and calcined at 500 C for 10 hours to prepare low-fired beryllium oxide according to the method of Spencer et al. (ref. 7).

A suspension of labelled oxide was prepared by combining 76.3 mg of the synthesized oxide with 5.0 ml of 0.9% sodium chloride and shaking overnight.

Rats were anesthesized with ether, placed in a glove box, the box sealed, and then either 0.15 ml or 0.3 ml of the labelled beryllium oxide suspension was administered intratracheally according to the method of Spencer (ref. 7). Rats were then removed from the glove box, allowed to recuperate for various time periods (30 min to 48 hours) and were then placed in the exposure chamber shown in figure 3.

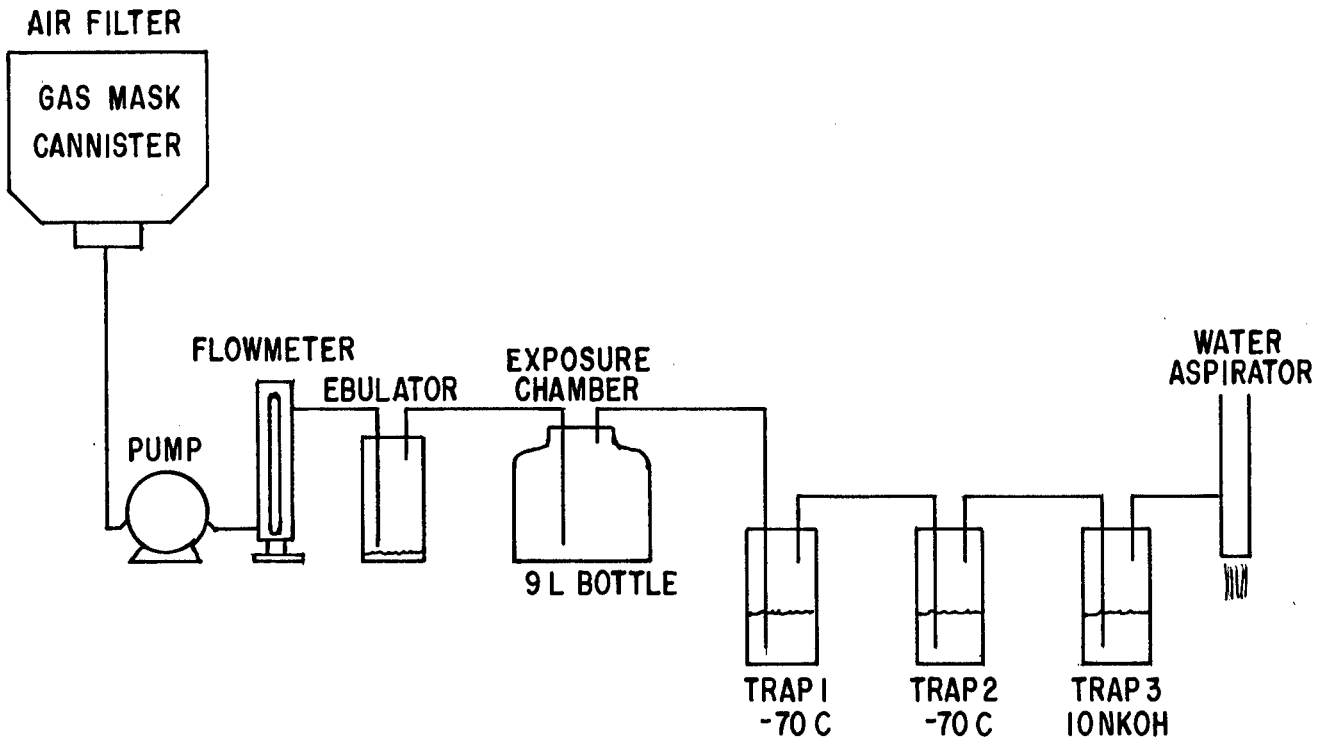

Figure 3. Experimental Exposure Arrangement

In the first series, rats 1 and 2 were each exposed for 30 minutes to a concentration of 4.4 and 9.7 mg trifluoroacetylacetone per liter. The exposure concentration was calculated by determining the weight of H(tfa) lost from the ebulator, calculating the volume of air pumped through the ebulator and simply dividing mg of H(tfa) by liters of air. In this series the amount of radioactivity in the traps was insignificant (see table XI).

In the second series, rats 3 and 4 were each administered 0.15 ml portions of the beryllium oxide suspension and then, after four hours, another 0.15 portion of the suspension was given to each animal. After a 48-hour recuperation period both rats were exposed to 11.7 mg/liter of H(tfa) for 30 minutes. The activity contained in the traps was not different from the first series (table XI). Gaseous hexafluoroacetylacetone (H(hfa) was also examined for its ability to remove beryllium from the lungs of rats. The compound was vaporized in the same way as described for trifluoroacetylacetone. Before working with ^{7}BeO treated rats, the acute effects of H(hfa) alone were evaluated. Four rats were exposed to vapors of H(hfa) in concentrations ranging from 4.1 mg/liter to 11.4 mg/liter for periods of 30 minutes. At these levels the compound produced apparently only acute irritation of mucous membranes and seemed to lack acute CNS effects.

Four rats were treated with 0.15 ml aliquots of the labelled—unlabelled BeO mixture and 24 hours later were exposed individually to H(hfa) vapors. Three of these rats were exposed to 4.4, 5.4, and 8.2 mg/liter H(hfa) for 60 minutes. The fourth animal was exposed to air only [no H(hfa)] at a flow comparable to that used in the previous exposures. Results obtained in these studies seemed to suggest that traces of labelled oxide were being leached out of the lungs of these animals. The three animals treated with H(hfa) exhaled small amounts (less than 0.1% of the dose given) of ^{7}BeO. No activity was obtained in the case of the air-treated animal.

These inhalation studies mark the first time that fluorinated b-diketones have been examined for their ability to chelate beryllium in vivo. The results obtained should be regarded as preliminary. While no encouraging results were obtained in these studies, some provocative results were seen with hexafluoroacetylacetone. Certainly more definitive studies employing fluorinated b-diketones (given by inhalation as well as other routes of administration) for the treatment of beryllium disease should be pursued in the future.

TABLE XI

REMOVAL OF BeO FROM LUNGS OF ANIMALS EXPOSED TO B-DIKETONE VAPORS

Experiment	*ml BeO Susp*	*Time (min)*	*Flow Rate (L min)*	*Ligand Conc. (mg/L)*	*Trap 1 & 2 Net cpm*	*Lung*	
						cpm	*mg BeO*
				H(tfa)			
Control	0	30	2.5	7.0	bkg	bkg	N.D.
Rat 1	0.3	30	2.5	4.4	17	29,000	1.58
Rat 2	0.3	30	2.5	9.7	16	29,570	1.60
Rat 3	0.3	30	2.5	11.7	15	27,563	1.51
Rat 4	0.3	30	2.5	11.7	15	25,440	1.42
				H(hfa)			
Rat 1	0	30	3.4	4.1	—	—	—
Rat 2	0	30	3.4	8.6	—	—	—
Rat 3	0	30	3.4	11.4	—	—	—
Rat 4	0.15	60	3.4	4.4	19	30,000	1.62
Rat 5	0	30	3.4	5.4	—	—	—
Rat 6	0.15	60	3.4	8.2	49	28,850	1.57
Rat 7	0.15	60	3.4	6.6	28	33,800	1.80
Rat 8*	0.15	30	3.4	—	0	33,400	1.77

N.D. = none detected

bkg = background counting level

* = no treatment

SECTION VII

MISCELLANEOUS IN VITRO STUDIES

A. Analysis Using Ammonium Trifluoroacetylacetonate [NH_4(tfa)] as a Chelating Agent.

In this section the development of a more sensitive method than any found thus far for the quantitative determination of beryllium in aqueous solutions is discussed. The new approach was conceived in an effort to try to find a simpler, more accurate method for determining beryllium in blood. Attempts to apply the method to the analysis of spiked blood samples, however, were either unsuccessful or at best mediocre.

1. Preparation of Ammonium Trifluoroacetylacetonate [NH_4(tfa)].—This compound was prepared by the gas phase reaction of trifluoroacetylacetonate with ammonia. Dry air was bubbled through an ebulator containing H(tfa) and the effluent passed into a 250 ml Buchner flask into which gaseous ammonia was also introduced. Immediately upon contact of the two reactants, fluffy crystals of NH_4 (tfa) form. The solid is collected and washed 4 or 5 times with dry benzene by suction filtration in order to remove unreacted (tfa). The product is allowed to air dry 5-10 minutes and then aqueous solutions of the desired concentration are made. The solid NH_4(tfa) is relatively unstable and will also sublime at ambient conditions. It must therefore be stored in sealed containers and resynthesized every two or three days.

2. Analysis—Four aqueous beryllium sulfate solutions were prepared with beryllium concentrations of 0.224, 0.022, 0.002, and 0.0005 μg Be/ml. Volumes of these solutions ranging from 0.1 ml to 0.5 ml were placed in silanized ampules and between 0.1 and 0.4 ml of an aqueous solution containing 2.48 mg NH_4(tfa)/ml was added. The ampules were centrifuged to assure complete removal of the reactants from the ampule walls and then 0.1-0.5 ml of benzene was added. The ampules were flame sealed, allowed to cool, then shaken vigorously and heated at 130C (ampules on sides on metal grid) for 10 minutes. The duration of heating is critical. After heating, the ampules were shaken, centrifuged 10 minutes and then analyzed gas chromatographically as reported before. Table XII gives the various combinations of reactants used and the results obtained.

TABLE XII

ANALYSIS OF AQUEOUS BERYLLIUM SOLUTIONS USING NH_4(tfa) AS A CHELATING AGENT

No.	*Volume Analyzed (ml)*	*Be Conc (μg/ml)*	*NH_4(tfa) (ml)*	*Benzene (ml)*	*Nominal Be Conc (pg/inj)*	*Chromat Peak Hgt (cm)*	*Calculated Be Conc (pg/inj)*
1	0.2	0.224	0.4	0.5	44	12.8	43.2
2	0.2	0.224	0.2	0.5	44	13.2	44.5
3	0.1	0.224	0.2	0.5	22	7.3	24.5
4	0.1	0.223	0.2	0.5	22	7.6	25.5
5	0.1	0.224	0.2	0.4	27	9.0	30.2
6	0.1	0.224	0.2	0.3	35	11.7	39.2
7	0.1	0.224	0.2	0.2	55	15.9	53.7
8	0.1	0.224	0.2	0.2	55	15.5	52.5
9	0.1	0.224	0.2	0.2	55	16.1	54.5
10	0.1	0.224	0.2	0.1	*44	12.5	42.2
11	0.4	0.022	0.2	0.5	**17	7.0	21.0
12	0.2	0.022	0.1	0.1	22	7.6	25.5
13	0.2	0.022	0.1	0.1	22	7.3	24.5
14	0.2	0.022	0.1	0.1	22	7.3	24.5
15	0.3	0.002	0.1	0.1	**7	1.9	6.2
16	0.4	0.002	0.1	0.1	**9	2.4	8.0
17	0.5	0.002	0.1	0.1	**10	3.1	10.2
18	0.5	0.0005	0.1	0.1	**2.5	***2.1	1.8
19	0.5	0.0005	0.1	0.1	**2.5	***1.6	1.5

All injections of 0.5 μl volume except * = 0.2 μl; ** = 1.0 μl.

Chromatograph attenuation = 4×10^{-9} amperes full scale (a.f.s.) except *** = 1×10^{-9} a.f.s.

Blood containing beryllium was analyzed in the same manner as reported above for the aqueous solutions. The results are given in table XIII.

TABLE XIII

ANALYSIS OF SPIKED BLOOD USING NH_4(tfa)

No.	*Blood Analyzed (ml)*	*Be Conc (μg/ml)*	*NH_4(tfa) (ml)*	*Benzene (ml)*	*Nominal Be Conc (pg/inj)*	*Chromat Peak* Hgt (cm)*	*Calculated Be Conc (pg/inj)*
1	0.2	0.045	0.1	0.2	22	0.75	2.5
2	0.2	0.045	0.1	0.2	22	2.5	8.2
3	0.2	0.045	0.1	0.2	22	0	0
Blood 4 not heated but let stand for 30 min at room temp with periodic shaking.							
4	0.2	0.045	0.1	0.2	22	2.8	9.2
4	0.2	0.045	0.1	0.2	22	3.0	10.0
4	0.2	0.045	0.1	0.2	22	3.5	11.7

*Chromatograph Attenuation was 4X.

3. These data indicate the approach using NH_4(tfa) as the chelating agent is excellent for the analysis of aqueous solutions of beryllium. No other procedure that has been tried in this laboratory is capable of detecting and quantitating such low levels of beryllium (less than 1 ppb). The outstanding feature of the procedure is the cleanliness of the aqueous samples (lack of electron capturing substances in the benzene layer), thus permitting operation of the chromatograph at very low attenuations. When applied to blood, the method proved to be incapable of detecting and quantitating low levels of beryllium (less than 0.05 ppm was not detected).

4. Attempts at Modification—Various attempts were made to modify the NH_4(tfa) method to make it acceptable for blood analysis and these are summarized below.

a. One milliliter of whole blood containing 0.1×10^{-6} g Be/ml was combined with 1 ml of concentrated nitric acid and this mixture was then heated at 130C in a vacuum oven until about 0.1 ml of yellow oil remained. This residual oil was extracted three times with benzene and then heated again in a vacuum oven to drive off excess benzene. The oil was combined with 0.9 ml 1M sodium acetate and 0.1 ml of NH_4(tfa) (2.5 mg/ml) and this mixture extracted with 0.2 ml benzene.

The beryllium concentration in the benzene layer was theoretically 100 picograms/μl. Chromatography of 0.5 ul aliquots of the benzene layer gave excellent quantitative results but also showed at least 10 interfering electron capturing impurities. The lowest usable attenuation was 8X.

b. Whole blood containing 0.1×10^{-6} g Be/ml was centrifuged and the plasma divided into two 0.2 ml fractions. One 0.2 ml aliquot was treated as indicated in table XIII for whole blood. The other 0.2 ml aliquot was combined with 0.4 ml of a 10% trichloracetic acid (TCA) solution, the mixture centrifuged, and 0.4 ml of the protein-free supernatant chelated and extracted as described in (1). This TCA treated sample gave totally unsatis-

factory results. The samples, when chromatographed, contained many electron capturing impurities and beryllium could not be detected. The analysis of the untreated plasma aliquot was more promising. About 50% of the beryllium (based on the assumption that *all* beryllium in the blood is located in the plasma) was detected and the sample was clean. This sample could have been run at an attenuation of 1×10^{-9} a.f.s.

c. A 0.6 ml aliquot of the buffered solution of residual oil from procedure (1) was taken to dryness in a 130C oven. The residue was ashed by heating at 500C for 1 hour. The ash was taken up in 0.6 ml of distilled water and then extracted and analyzed as described in (1). The samples were clear but beryllium was not detected.

d. More extensive ashing studies were undertaken in which NH_4(tfa) was employed to chelate beryllium subsequent to ashing. Blood and plasma samples containing ionic beryllium were placed on silanized glass fiber filter discs and ashed at 500C for various time intervals varying from 15 minutes to 12 hours. The discs were then placed in ampules along with aqueous NH_4(tfa) and benzene and heated. Chromatography of aliquots of the benzene layer gave either extremely dirty samples or poor quantitation.

Additional ashing studies were performed in which chemical ashing was employed in conjunction with thermal ashing. Four centimeter glass fiber discs were each moistened with 0.2 ml of blood containing 8.96×10^{-8}g Be/ml. As soon as the blood soaked into the filter paper, 10-15 drops of concentrated nitric acid were added and the filter paper discs were heated at 500C for 15 minutes. This nitric acid plus heat treatment was repeated two additional times. Following the third treatment the discs were almost entirely white with only a slight brown residue at the periphery. These discs were then placed in ampules along with 0.2 ml of aqueous NH_4(tfa) (2 mg/ml), 1.0 ml distilled water, and 0.5 ml benzene. Extraction of the discs followed by analysis of the benzene layer gave no indication of beryllium.

5. Further efforts to employ NH_4(tfa) to chelate beryllium in whole blood employed either 50 μl or 200 μl of blood, 0.2 ml benzene, and 0.1 ml of NH_4(tfa) (2 mg/ml). These reactants were sealed in ampules and shaken at room temperature for time intervals varying from 10 min to 40 min. In general, poor results were obtained — cleanliness of samples and quantitation were unacceptable.

Although the analysis of aqueous samples containing as little as 0.0005×10^{-6}g Be/ml was remarkably successful using NH_4(tfa), this approach does not appear suitable for biological materials. Efforts to modify the procedure including chemical and thermal ashing steps proved unfruitful. Thus attempts to use NH_4(tfa) in quantitation of beryllium in blood were discontinued.

B. Attempts to Use Extraction and Chelation for Analysis of Beryllium in Blood.

The method of Ross and Sievers (ref. 3) for quantitative extraction and analysis of aqueous beryllium solutions was also examined to determine if this procedure could be applied to the quantitation of beryllium in blood. Initially, the method of Ross and Sievers was applied, with only minor modifications, to the analysis of aqueous beryllium samples. Good analytical results were obtained when an aqueous solution containing 4.48×10^{-6}g Be/ml was analyzed. However, the NH_4(tfa) procedure appears to be better—it is simpler and faster, yet is accurate and precise at beryllium levels greater than 4.48×10^{-6}g Be/ml.

The method of Ross and Sievers when applied to the analysis of blood proved to be unsatisfactory. Details of this unsuccessful attempt are presented below:

Two-tenths millimeter of an aqueous beryllium stock solution containing 11.2 mg Be/50 ml was placed in a 10 ml volumetric flask and heparinized whole blood added to make 10 ml, resulting in a Be concentration of 4.48×10^{-6}g Be/ml.

Four 1.0 ml aliquots of the spiked blood were placed in silanized, screw cap culture tubes. To two tubes were added 1.0 ml of 1 M acetate buffer and 1.0 ml of 0.0005 M trifluoroacetylacetone in benzene. To the other two tubes 1.0 ml benzene and 0.2 ml of 2 mg NH_4(tfa)/ml solution were added. All tubes were shaken for 1 hour.

After shaking, the tubes were centrifuged for 5-10 minutes and 1 μl aliquots of the benzene layer from the NH_4(tfa) tubes were analyzed. Interference-free results were obtained but beryllium quantitation was poor.

The 0.0005 M H(tfa) tubes were treated with 20 μl of 28% NH_4OH, recentrifuged and aliquots of the benzene layer chromatographed. Interfering substances were encountered which prevented accurate quantitation of beryllium. These efforts did not yield promising results and were not pursued further.

SECTION VIII

GENERAL OBSERVATIONS AND COMMENTS

Beryllium analysis by chelation and gas chromatography is remarkably simple. However, experience has taught us some things that are extremely important to the worker in this field.

Beryllium trifluoroacetylacetonate is very volatile. This material will sublime at ambient conditions. This was learned when brief attempts were made to subject benzene solutions of $Be(tfa)_2$ to thin layer chromatography. We found that this method of separation was totally unsatisfactory due to the loss of $Be(tfa)_2$ by sublimation. The volatility of $Be(tfa)_2$ requires that the ampules be sealed.

$Be(tfa)_2$ is unstable in the presence of base. For this reason we have always used NH_4OH in preference to NaOH. It was found that $Be(tfa)_2$ in benzene degraded rather quickly in contact with aqueous NaOH, yet in contact with NH_4OH, degradation was slow. This indicates that ampules should be analyzed as soon as possible and not be allowed to stand more than 30 minutes after centrifugation.

Apparently the type of glass from which the ampules are fabricated influences analytical results. In our work we used ampules from several sources—ampules made from diSPO® pipettes, ampules from the VIRTIS Company and others. We tried using silanized ampules and unsilanized ampules. Surprisingly, treatment with the silylating reagents ($(CH_3)_3SiCl$, hexamethyldisilizane, and $(CH_3)_2SiCl_2$) did little to influence quantitative results, but the type of glass certainly seemed to have a marked influence. We had little or no success when any ampule except that fabricated from diSPO® Pipettes was used.

Beryllium, according to the literature, is present in minute amounts in the atmosphere and earth's crust. In our experience we found that this metal is, in the materials we were concerned with, present in amounts which are less than 10 ppb (10×10^{-9} g/g) in some cases and less than 1 ppb in most cases. So-called background amounts of beryllium were seldom encountered in our reagents or the tissues from control animals. The source of "spurious" beryllium was most often found to be the result of "cross-contamination" of our own glassware and reagents.

We routinely washed our glassware in Alconox using tap water and rinsed with ordinary distilled water. For extremely low-level analysis, glassware was treated with aqua regia following washing, and then treated overnight with a silylating reagent (10% HMDS in $CHCl_3$). The silanized glassware was oven dried and was then considered ready for use.

Analysis of blood samples is most successful when the blood is fresh. If this is not possible, the blood should be kept at 5C until analysis can be performed. Frozen blood when thawed yields a heterogeneous mixture of denatured blood proteins which is difficult to pipette and may lead to erratic results. Blood at room temperature decomposes and the low-molecular weight decomposition products tend to give baseline rise during chromatography. Organs and tissues for analysis can be frozen prior to analysis with good results.

Electron capture gas chromatography is nearly impossible to perform unless one has an instrument which was designed for this purpose. Electrical and pneumatic performance characteristics must be characteristically stable. When excess, unreacted H(tfa) is likely to be present in samples, hot metal surfaces as may be present in the injection port, column, or detector are objectionable. Glass columns (or possibly Teflon®) and glass-lined injection ports are mandatory. Column preparation is important. We tried a number of stationary phases, including OV-17, SE-30, QF-1 and SE 52, and various supports including Gas Chrom Q, Anakrom ABS, and glass beads. We found none of these to perform as well as 10% SE 52 on Gas Chrom Z. In general the column should be moderately tightly packed (stationary phase coated on support by careful application of the rotary flash evaporator method) in a silanized glass column and conditioned as follows:

30 min @ room temperature, no flow

1 hour @ 100C with 1cc/min flow

Overnight @ 150C with 1cc/min flow

Eight hours @ 180C with 30 cc/min flow

Eight hours @ 110C with 100 cc/min flow

This conditioning is followed by injection of standard solutions of $Be(tfa)_2$ in benzene. The peak size and shape improve with repeated injections and an optimum is usually reached after 5-10 injections. Poor peak shape at this point indicates usually an inadequate preparation of the column. During use of the column, it may be necessary to rejuvenate the column in situ. This can be done by injecting 1-10 μl of H(tfa) or by injecting 10-100 μl HMDS into the column. This drastic treatment will obliterate the sensitivity of the detector for 3 to 24 hours but usually will improve column performance. Depending upon the number of analyses, a new column should be fabricated every 6 to 12 months.

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