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Title: GAS CHROMATOGRAPHIC DETERMINATION OF SELENIUM IN BIOLOGICAL MATERIALS

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A method for the determination of selenium in biological materials by gas-liquid chromatography has been developed and successfully applied to kidney, heart, liver, and muscle tissue. The initial digestion of animal tissue was accomplished in a mixture of nitric and perchloric acids (3:1) until "water whiteness" of solution was achieved. It was found that an additional two to four hours severe heating was necessary to destroy the last traces of organic matter. No selenium was lost in this process but some was oxidized in Se(VI) and had to be reduced by acid refluxing in strong HCl. The selenite was quantitatively complexed with 4-chloro-o-phenylenediamine at pH = 1.0 to 1.5. The 5-chloropiaselenol formed was extracted into one ml of toluene and chromatographed on 3% OV-17 on Chromosorb G at 180°C, using an electron capture detector maintained at 200°C. Tissue samples containing 0.07 to 1.0

micrograms selenium were analyzed and the selenium content determined with a relative standard deviation of 3.5% which is comparable to other methods of selenium determination.

Gas Chromatographic Determination of Selenium in Biological Materials

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDIX FIGURES

LIST OF TABLES

GAS CHROMATOGRAPHIC DETERMINATION OF SELENIUM IN BIOLOGICAL MATERIALS

INTRODUCTION

The study of selenium both from a nutritional and industrial standpoint has created a need for better, faster, and more sensitive methods for its analysis. In the past decade numerous methods have been published on selenium determination all of which suffer from one or more shortcomings. The excellent reviews of Watkinson (1967), and Rosenfeld and Beath (1964) give good summaries and bibliographies on the various techniques and should be consulted for particulars.

The application of gas-liquid chromatography to selenium determination has only recently been suggested which accounts for the paucity of reports along this line of research. Evans and Johnson (1966) first reported the GLC of selenium compounds. Using a flame ionization detector (FID), they chromatographed several purified alkyl selenides dissolved in carbon disulfide. However, this procedure was unsuitable for submicrogram analysis for many reasons. Among them was the difficulty in forming the compound (recoveries of 40 to 80% were reported) and the large amount of compound needed for detection (at least 0.1 milligram selenium).

The work of Nakashima and Toei (1968) established the

feasibility of the gas chromatographic method in selenium analysis. Standard solutions of selenium (IV) were quantitatively complexed with 4-chloro-o-phenylenediamine and, after toluene extraction, the complex was chromatographed on a 4 mm X 2 meter glass column of 15% SE-30 on Chromosorb W. The chlorine on the 5-chloropiaselenol allowed determination by electron capture detector. Their results showed that as little as 0.08 microgram selenium could be detected, an amount approaching the desired sensitivity needed in selenium analysis.

Shimoishi and Toei (1970) published the first, and as yet, only application utilizing gas chromatography by determining the selenium content in pure sulfuric acid. They used a bromine-bromide redox buffer to convert selenium (0) in sulfuric acid to selenium (IV) which was complexed with 4-nitro-o-phenylenediamine and extracted into toluene. Their results show a sensitivity greater than that reported originally by Nakashima using the chloro-substituted diamine.

This method of Shimoishi and Toei seems to be applicable only to homogeneous samples containing elemental selenium. Their oxidative process (the bromine-bromide redox buffer) is obviously not suitable for destruction of organic material which must be accomplished in biological tissues to liberate the selenium. Therefore, a digestion step had to be incorporated into the procedure.

The successful analysis of a complex matrix such as biological

tissue would accomplish two things. It would provide a generalized procedure which could be applied to other types of samples as well and also elevate the gas chromatographic procedure to an equal par with other well known, established techniques.

The gas-liquid chromatographic method is attractive for routine analysis for it possesses other favorable advantages in addition to the already established sensitivity. Wet digestion of organic materials can be used which aids in making the procedure more rapid. The selenium content is determined on a discrete species, the selenium-diamine complex, which is separated by the chromatographic column before detection. Any potentially interfering materials also extracted into the organic phase are thus eliminated. The volume of toluene extract injected onto the column can be chosen to give the desired peak height of the 5-chloropiaselenol. Samples containing either very small or very large amounts of selenium can be successfully analyzed without the need to repeat the digestion with a different size sample. The length of time between the extraction and the chromatography of the toluene is not as critical as, for example, with the fluorometric method in which the fluorescence must be measured within four hours after extraction. Also, the chromatogram can serve as a permanent record of the analysis. These advantages gave impetus to the development of this new procedure.

MATERIALS AND METHODS

The following is a description of the actual procedure developed for the analysis of selenium as a result of this research. It is presented here as an overall summary of the method which will be explained in detail in the Results and Discussion section.

Sample Digestion

Wet digestion of biological materials was accomplished using a mixture of nitric and perchloric acids (3:1; 15 mls of mixture per gram dry weight of material). The digestions were carried out in either 100 ml kjeldahl flasks or 50 ml erlenmeyer flasks, both vessels giving equally good results when used with properly standardized procedures.

The 100 ml kjeldahl flask digestions were performed on a rack equipped with adjustable gas burners. The necks of the flasks were inserted into the openings in a glass manifold from which the air was being slowly withdrawn via a water aspirator to reduce the amount of acid vapors in the fume hood.

The use of 50 ml erlenmeyer flasks allowed many more samples to be digested simultaneously and conveniently, provided caution was used during the digestion. Due to the small size of the flasks the wet oxidation was carried out gently to avoid loss of

sample making the procedure more time consuming. It was found that an electric hot plate with heat adjustment capabilities provided the necessary control over the digestion. The necks of the small flasks were fitted with a small funnel (i. e., a 25 mm filtering funnel) which acted as a cold finger for refluxing the acids. The samples were pre-digested (tissues allowed to soak in the cold acid mixture) 24 hours to lessen the danger of sample loss due to foaming upon initial heating. The heat was then adjusted to provide for gentle heating until the digest turned "water white" and all the nitric acid was boiled off.

The acid digests were heated until dense perchloric acid fumes appeared and the solution became "water white" or nearly so upon cooling. The samples were then refluxed under more severe heat for a time dependent on the amount of sample used, usually two to four hours, causing the destruction of the remaining organic materials. If extra perchloric acid was added during the digestion process, it could be boiled off at this time. However, the flask should not be allowed to go dry.

To the cooled acid digests 10 mls of 1:1 HCl was added and the samples refluxed for one hour, after which the excess HCl was driven off. It was found that one hour was ample time to reduce the selenate to selenite.

Complexation and Extraction of the Selenium

The reagent, 4-chloro-o-penylenediamine, as received from the supplier was found to be the brownish-red color of the oxidized, photodecomposed reagent. Attempts to re-crystallize the reagent were far from successful. However, by dissolving the reagent in 1 N HCl (also 1% in $NH_2OH \cdot HCl$), filtering it into a separatory funnel, and extracting the aqueous phase with toluene, a fairly clean solution of the reagent was obtained.

The acid digests or standardized selenious acid solutions were quantitatively transferred to a 125 ml separatory funnel with deionized water and 1 N HCl. Two mls of 2.6M formic acid were added and the pH adjusted to between 1 and 1.5 with ammonia solution (1:1). Five mls of 0.1M EDTA were added and the volume diluted to 50 mls (volume marked on the separatory funnel). Two mls of the freshly prepared 0.5% complexing reagent were added, the complexing was allowed to proceed for 1.5 hours, and the 5-chloropiaselonel was extracted into one ml of toluene by mechanical shaking for five minutes.

Gas Chromatography of the Piaselenol

The piaselenol was chromatographed on a 1/8 inch O.D. X 6 foot stainless steel column packed with 3% OV-17 on Chromosorb G,

HP while maintaining the oven temperature at 180° C. The injection port was maintained at 210° C and the detector was 200° C. The electron capture detector had tritium as the beta source and 90 v. d. c. as the collector voltage. With the above conditions and a flow rate of about 35 ml/minute, the piaselenol was eluted in about four minutes (Figure 3).

Use of Radiotracer(Se-75) Sodium Selenite

The radioisotope Se-75 as sodium selenite, a gamma emitter, was of invaluable aid in ferreting out the problems of the method, specifically, the digestion step. The aqueous phase from tissue samples and standards alike, which had previously been spiked with this isotope, was monitored for radioactivity at various stages during the procedure. The advantage lay in the fact that the selenium could be followed from the digestion through the complexation and extraction on the same sample. The loss of selenium, if any, could be easily determined and the troublesome part of the procedure identified. This technique actually led to the discovery that the selenite was being oxidized to selenate as will be explained later.

Sources of Error in Using the Radiotracer

The radioactivity was monitored and quantitated in a gamma spectrometer equipped with a well-type detector using a thallium

activated sodium iodide crystal. The instrumental parameters were chosen so as to minimize the counting error to one percent (accumulation of 10, 000 counts or ten minute counting period) per sample (Wang and Willis, 1965).

In using the well-type detector, sample geometry plays an important role in obtaining accurate, reproducible count rates. This source of error was minimized by attempting to count identical volumes of liquid. The usual procedure followed was to quantitatively transfer the liquid, either the aqueous or organic phase, to a volumetric flask, dilute to volume, and count aliquots of the same size. With aqueous samples the aliquots were transferred directly to the 15 X 150 mm polystyrene counting tubes and counted. However, the toluene had to be placed in stoppered glass test tubes (7.5 x 15 mm) which were then placed in the polystyrene tubes to be counted.

In comparing the count rates of duplicate aqueous samples, with and without the glass test tubes, differences of three to five percent were observed indicating that the glass tube had a slight quenching effect. The majority of this difference was indeed due to quenching and not to geometric factors.

The importance of the above fact is stressed here because it explains the lower values for the extraction efficiency seen in Table 1 (column 5). The actual efficiency is about 83% (see p. 18) as

determined by counting both the initial and final radioactivity in the glass test tubes.

RESULTS AND DISCUSSION

The destruction of organic material by wet digestion was selected because of its speed and ease of sample preparation. Gorsuch (1959) reported excellent recoveries of selenium after digestion with mixtures of nitric-perchloric-sulfuric acid and nitric-perchloric acid. The nitric and perchloric acid mixture (without sulfuric acid)was chosen due to the possible interference of the sulfate anion in the selenium-diamine complex formation as reported by Tanaka and Kawashima (1965).

Examination of the Wet Digestion Step

Initial Problem of Digestion

At the onset of the study the digestions were carried out until the solutions were "water white" or nearly so which traditionally signalled the completion of the digestion. However, when the selenium was complexed and the toluene extract chromatographed, the output showed the presence of many unidentified components which were not complexed by the reagent, but were extracted with the selenium diamine complex. The above was proven true as shown by the following example. A 1.507 gm wet weight liver sample, after digestion to perchloric acid fumes and solution "whiteness", was

processed as described in the Materials and Methods section. The solution was extracted with two mls of toluene just prior to adding the complexing reagent. The reagent was then added, the complexation allowed to proceed for 1.5 hours, and the extraction accomplished into one ml of toluene. The chromatograms of the initial toluene extract (Figure 1) and of the toluene extract after complexation (Figure 2) indicate the presence of a tremendous amount of material which is detectable by EC. The selenium-diamine complex peak which elutes at 4.9 minutes (marked by the arrow) was completely inundated by this extraneous organic material. Therefore, this material had to be removed in order to successfully use wet digestions.

Obtaining "Chromatographically" Clean Digests

It was discovered that these components in the acid digest could be destroyed or volatilized by more severe heating in perchloric acid after the "water whiteness" was achieved rendering them unextractable into the toluene phase. The period of time used for further digestion was governed by the type and amount of tissue sample. Generally, it was found that a three to four hour extended digestion time was needed for a three gram wet weight liver sample. Liver samples proved to be the most difficult tissue to digest completely and

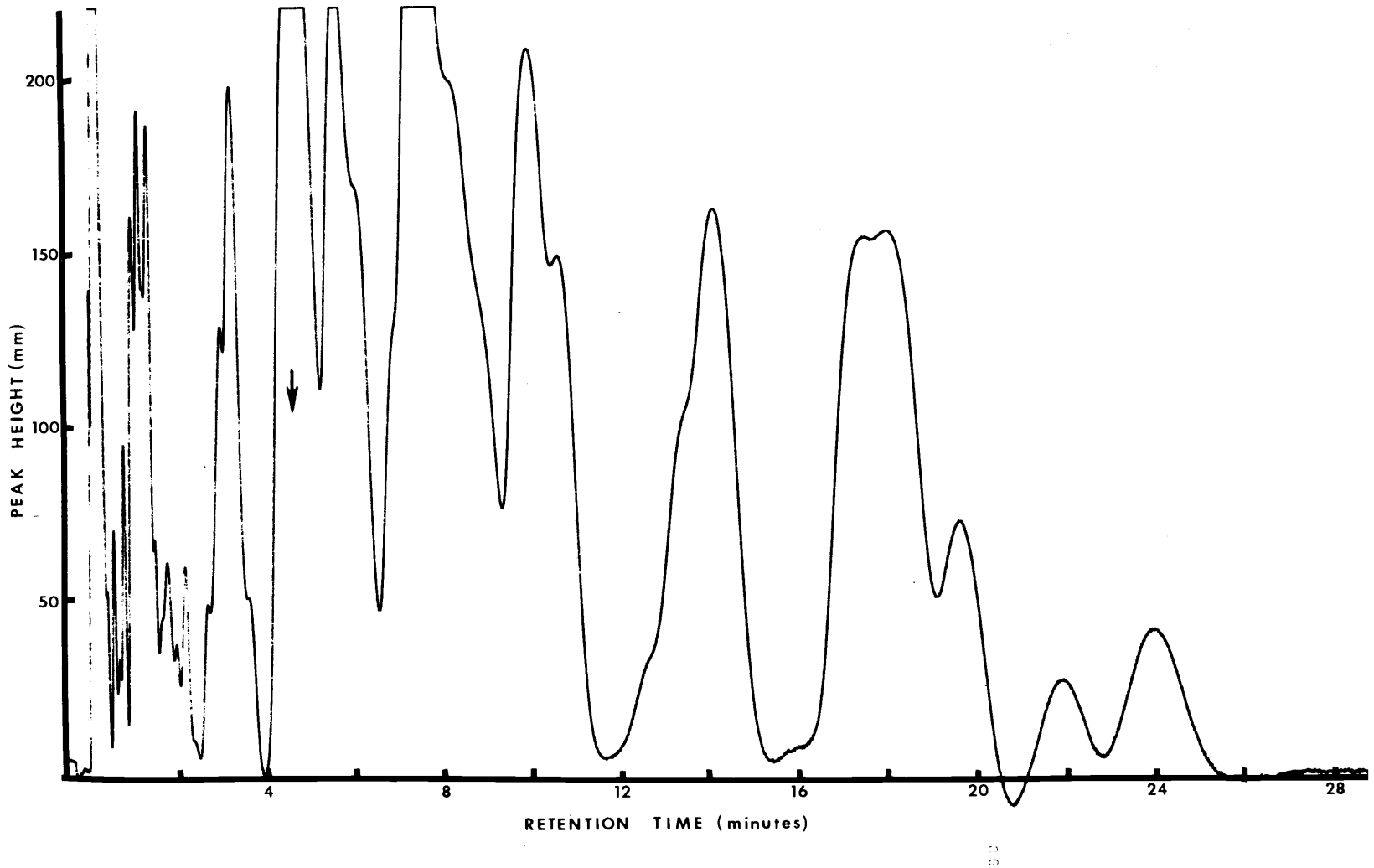

Figure 1. Chromatogram of the toluene extract of a lamb liver digest before complexation.

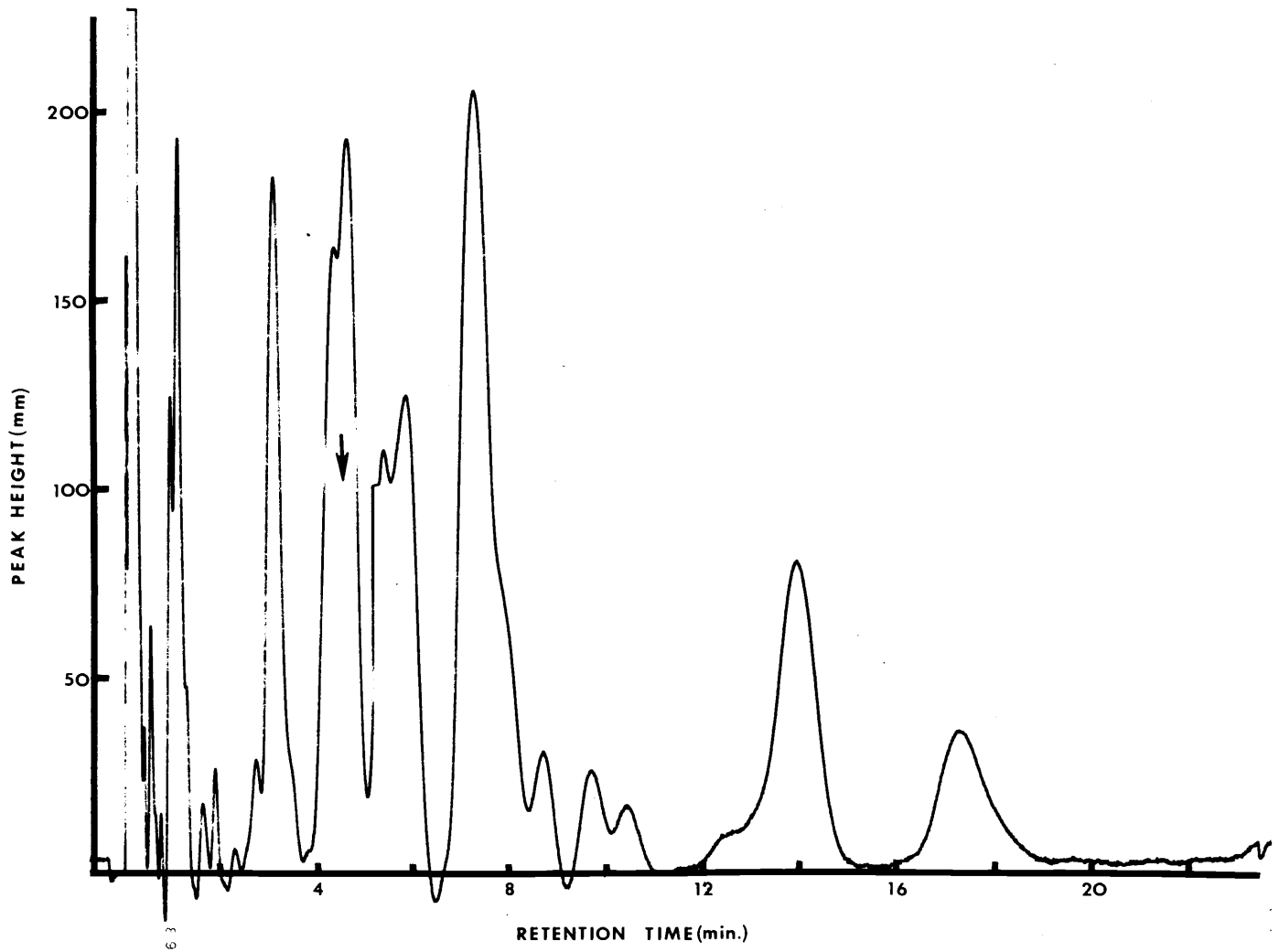

Figure 2. Chromatogram of the toluene extract of the liver digest after complexation.

for this reason was used as a sample matrix in many of the experiments.

Solving the Selenium (IV)-(VI) Problem

The increased digestion time solved the problem of obtaining clean digests, but created another. As could be expected, the severe oxidizing conditions of hot perchloric acid also converted the selenite species to selenate which does not form complexes with aromatic ortho-diamines. The selenium (IV) to (VI) conversion was confirmed by comparing the recovery of radioactivity in duplicate digested and non-digested samples (containing Se-75 sodium selenite) following precipitation with barium chloride (Table 1). Even though barium selenate is far from being quantitatively precipitated, a relative increase in radioactivity of ten-fold compared to non-digested blanks was observed (Table 1, column 4). Sodium sulfate was used to aid in the collection of the precipitate which accounts for residual radioactivity in the non-digested samples. To show that the low recovery was not due to loss upon digestion, an aliquot of one duplicate digest was counted before the precipitation step which showed a 100% recovery of the radioselenium.

Since the formation of selenate is concomitant with the production of a clean digest, the reduction to selenite must be made. Refluxing of selenic acid in dilute hydrochloric acid below 100° C will

Table 1. Recovery studies using Se-75 sodium selenite.

Sample designation	Recovery of radioactivity (counts per minute)			
	Initially added[a]	After digestion (%)	$BaSeO_4$ ppt. (%)	5-Chloropiaselonel extracted into toluene (%)
Barium selenate precipitation.				
1. Standard (not digested)	36, 100		1, 065(3)[b]	
2. Standard (digested)	36, 100		16, 085(44)[b]	
Evaluation of HCl refluxing using lamb tissues.				
3. Standard (HCl refluxed)	36, 100	37, 000(102. 5)		28, 550(79. 1)
4. Standard (not digested)	36, 100			27, 350(75. 8)
5. Liver a. 3. 120 gms.	36, 100	35, 700(98. 9)		400(1. 1)
b. 3. 047 gms. (HCl refluxed)	36, 100			28, 550(79. 1)
6. Kidney a. 1. 580 gms.	36, 100	35, 000(97. 0)		5, 650(15. 6)
b. 1. 681 gms. (HCl refluxed)	36, 100			31, 000(85. 9)
7. Muscle a. 5. 232 gms.	36, 100	37, 862(104. 9)		6, 220(17. 2)
b. 5. 330 gms. (HCl refluxed)	36, 100			26, 600(73. 7)
Use of HCl refluxing on digested standard and samples.				
8. Standard	33, 900			27, 490(81. 1)[b]
9. Liver a. 3. 909 gms.	33, 900			27, 490(81. 1)
b. 4. 015 gms.	33, 900			27, 570(81. 3)
c. 4. 140 gms.	33, 900			27, 340(80. 6)
d. 5. 210 gms.	33, 900			27, 880(82. 2)

a. Each addition of the radioactive stock solution contained 0. 065 microgram selenium as sodium selenite. b. Average of duplicate samples.

accomplish this reduction,

$$H_2SeO_4 + 2\,HCl \longrightarrow H_2SeO_3 + H_2O + Cl_2.$$

However, it was also reported that loss of selenium as the volatile chlorides and reduction to the element can take place (Bagnall, 1966). It will be shown that the presence of hot perchloric acid reduces this possibility greatly, if not completely.

Items three thru seven (3-7) in Table 1 were included to substantiate the aforementioned procedure. Three tissue samples in duplicate and a blank were spiked with Se-75 sodium selenite prior to digestion. One duplicate of each tissue sample and the blank were monitored after the severe digestion to determine the recovery of radioactivity (Table 1, column 3) showing the percent recoveries in parenthesis. The recovery is quantitative, supporting the work of Gorsuch, even after prolonged heating. The same blank and the other duplicate tissue samples were refluxed in about 3N HCl for two hours. All samples were then complexed and extracted with the amount of radioactivity recovered in the toluene phase listed in column 5 (Table 1). By comparing the samples with and without HCl acid refluxing it can be readily seen that the acid refluxing does perform the desired reduction. The variation in the extraction efficiencies can only be explained by procedural errors. This data was included primarily to point out the effect of the HCl refluxing.

In addition to the radiotracer data, the aqueous phase of these same tissue samples were extracted into one ml of toluene immediately before complexing reagent addition. The chromatograms of these extracts showed no peak in the region of 5-chloropiaselenol elution.

The procedure was again tried with four liver samples and two standards spiked with Se-75 sodium selenite prior to digestion. The data in items eight and nine (8-9) in Table 1 give the recovery of radioactivity in the toluene after extracting the piaselenol. The agreement of the extraction efficiencies of the standards and samples substantiates the validity of HCl refluxing. Therefore, the loss of selenium as reported by Bagnall seemingly does not occur. Both the presence of the perchloric acid and the slow refluxing must indeed moderate the reduction.

Study of the Extraction Procedure

Effect of Volume of Aqueous Phase

Radiotracer Se-75 sodium selenite was used again to examine the extraction procedure. Samples were prepared using identical amounts of isotope in different volumes of aqueous phase. After complexation the 5-chloropiaselenol was extracted into one ml of toluene which was counted. The radioactivity in the toluene as shown in

Table 2 indicates little difference in the values except for two: one each at 37 and 67 mls of aqueous phase. In any case an error of five mls either way in adjusting the volume to 50 mls will have a negligible effect on the amount of selenium extracted.

The data in Table 2 can be used to evaluate the extraction coefficient of the 5-chloropiaselenol from the aqueous phase into one ml of toluene. By taking the average of the six recovery values corresponding to extractions from 47, 52, and 57 mls of aqueous phase (24,930 cpm), 83% of the selenium as the complex is being transferred into the toluene phase in just one extraction. This represents an extraction coefficient of about 265. It will be reiterated that 83% is the extraction efficiency and the values obtained previously (Table 1, column 5) are about three to four percent low due to errors in counting technique.

Effect of Sample Size

In addition to the volume of aqueous phase, the amount of complex extracted was also studied. The results in Table 3 show that amounts of selenium (IV) of at least one microgram can be extracted into one ml of toluene.

Chromatograms of the 5-Chloropiaselenol

The form of the gas chromatographic output appears in Figure 3.

Table 2. Effect of volume of aqueous phase on extraction.

Volume(ml)[a]	Radioactivity in toluene extract(counts per minute)[b]
37	21, 530
37	24, 500
47	24, 750
47	25, 710
52	24, 810
52	24, 200
57	25, 900
57	24, 130
67	23, 730
67	24, 500

[a] Each sample contained 0. 50 micorgram selenium as unlabeled sodium selenite.

[b] Each sample contained 29, 775 counts per minute before extraction (0. 065 microgram of radiotracer selenium).

Table 3. Effect of amount of piaselenol on extraction.

Unlabeled selenium added(microgram)	Labeled selenium[a] added(microliter)	Radioactivity in[b] toluene extract(cpm)
0.15	25	15,460
0.40	50	30,035
0.75	75	47,050

[a] Volume of stock solution of radiotracer Se-75 sodium selenite added. Each 25 microliters contain 0.05 microgram selenium as labeled sodium selenite.

[b] Each value is the average of duplicate samples.

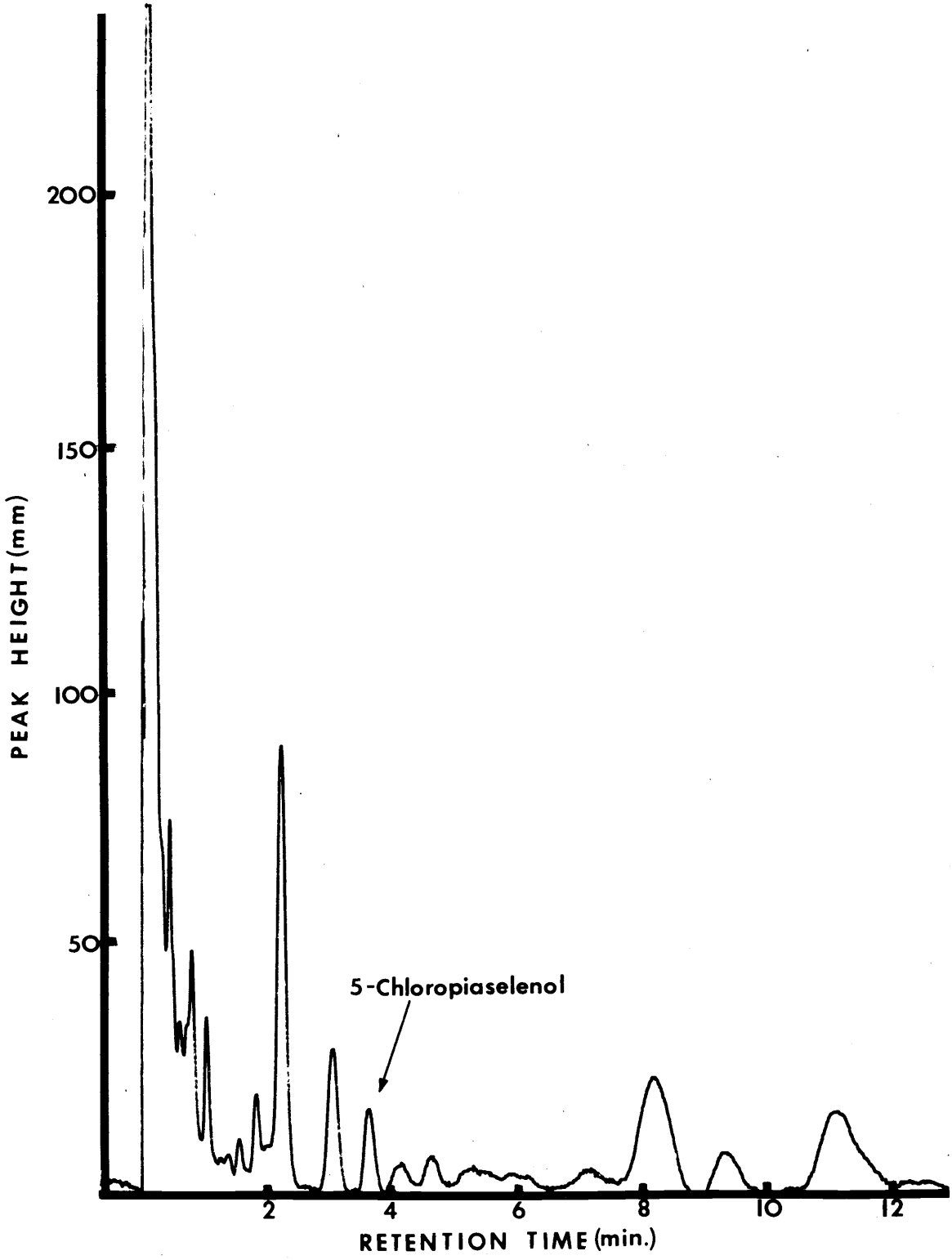

Figure 3. Chromatogram from the 0.05 microgram selenium standard.

It represents a four microliter injection of toluene extract from a 0.05 microgram selenium standard as selenite in 50 mls of solution. The height of a 5-chloropiaselenol peak (eluted at 3.9 minutes) is an indication of the possible sensitivity using the GLC method. The three peaks at longer elution times (after the selenium-complex peak) are due to decomposition products in the reagent since they appear in standards and samples both.

A calibration curve was constructed using five replicate standards for each data point (Figure A-1) including also the magnitude of the standard deviation. It appears that the calibration curve is linear up to the 0.25 microgram selenium standard after which it curves slightly indicating an exponential-type relationship, which cannot be caused by column overloading, because the plot of peak area (peak height times width at half height) versus amount selenium also shows this curve. However, the curve can be conveniently constructed and used if standards in the appropriate concentration range are selected. Since the sensitivity is good, samples can be chosen to contain 0.05 to 0.30 micrograms selenium which will fall in the linear range of the calibration curve.

Two examples of the gas chromatographic output of the toluene extract from tissue samples appear in the appendix. The chromatogram from a 1.3 ml whole blood digest appears in Figure A-2, while Figure A-3 is representative of a 0.486 gm dry weight kidney digest.

The concentration of selenium in the above kidney and blood were 0.472 and 0.183 ppm, respectively.

Evaluation of the Method Using Standard Additions

Kidney and heart tissues from lambs were used to evaluate the technique by standard additions. In order to assure representative sampling, the tissues were homogenized in a blender with deionized water and delivered to the digestion flask via pipet. They were then dried in a vacuum oven at 22° C. Even though the heart tissues were not dried after five days, they had attained a stable weight. Two of the four samples of each tissue were spiked with 0.50 microgram of selenium as sodium selenite after which they were processed as described in the Materials and Methods section. The vital statistics of the analysis appear in Table 4 along with the peak heights for the standards (Figure 4) which were carried through the same procedure. The values in column 4 (total selenium) for the spiked samples were obtained by subtracting 0.50 micrograms from the total selenium value read on the calibration curve corresponding to the peak height in column 3. The recoveries on these three tissues show good precision as indicated by the percent relative standard deviation.

Table 4. Results of the analysis of biological materials.

Tissue weight (grams)	Added selenium (micrograms)	Peak height (mm)	Selenium found (micrograms)	ppm[a]
Lamb #85 kidney				
0.456	none	79	0.46	1.01
0.480	none	82	0.49	1.02
0.464	0.50	134	0.48	1.03
0.500	0.50	142	0.56	1.12
	Relative standard deviation = 4.85%			
Lamb #83 kidney				
0.445	none	73	0.41	0.922
0.486	none	73	0.41	0.843
0.472	0.50	129	0.43	0.913
0.463	0.50	128	0.42	0.903
	Relative standard deviation = 3.98%			
Lamb #83 heart				
6.590	none	13	0.070	0.0106
6.073	none	12	0.063	0.0104
6.344	0.50	90	0.064	0.0101
6.008	0.50	90	0.064	0.0106
	Relative standard deviation = 2.27%			
Standards				
	Blank	0		
	0.100	19.5		
	0.250	47		
	0.500	83		
	0.750	111		
	1.00	137		

[a] Variance (12 normalized values): $s^2 = 0.001217$

Analysis of the Error in the Procedure

The total variance of the method can be considered to be the sum of the instrumental variance (due to injection and instrumental parameters) and the chemistry variance (digestion, complexation and extraction parameters), or

$$s^2_{total} = s^2_{instrumental} + s^2_{chemistry}.$$

The data in Table 4 was used to evaluate the total variance by normalizing the ppm values for each tissue with its respective average. The twelve normalized values were pooled and subjected to statistical analysis using the O. S. U. Computer Center's program *SDD (Simple Data Description) yielding a variance corresponding to 3.5% relative standard deviation. This relative standard deviation for the overall method is comparable to other methods of selenium analysis.

The variance due to instrumental parameters was obtained from the normalized peak heights of ten consecutive injections of the toluene extract from the 0.50 microgram selenium standard (Table A-1). The relative standard deviation of the instrumental parameters amounts to 3.3%. An F-test shows that the difference between the variances is insignificant at the 0.1 level, indicating that non-instrumental sources of indeterminate error are negligible.

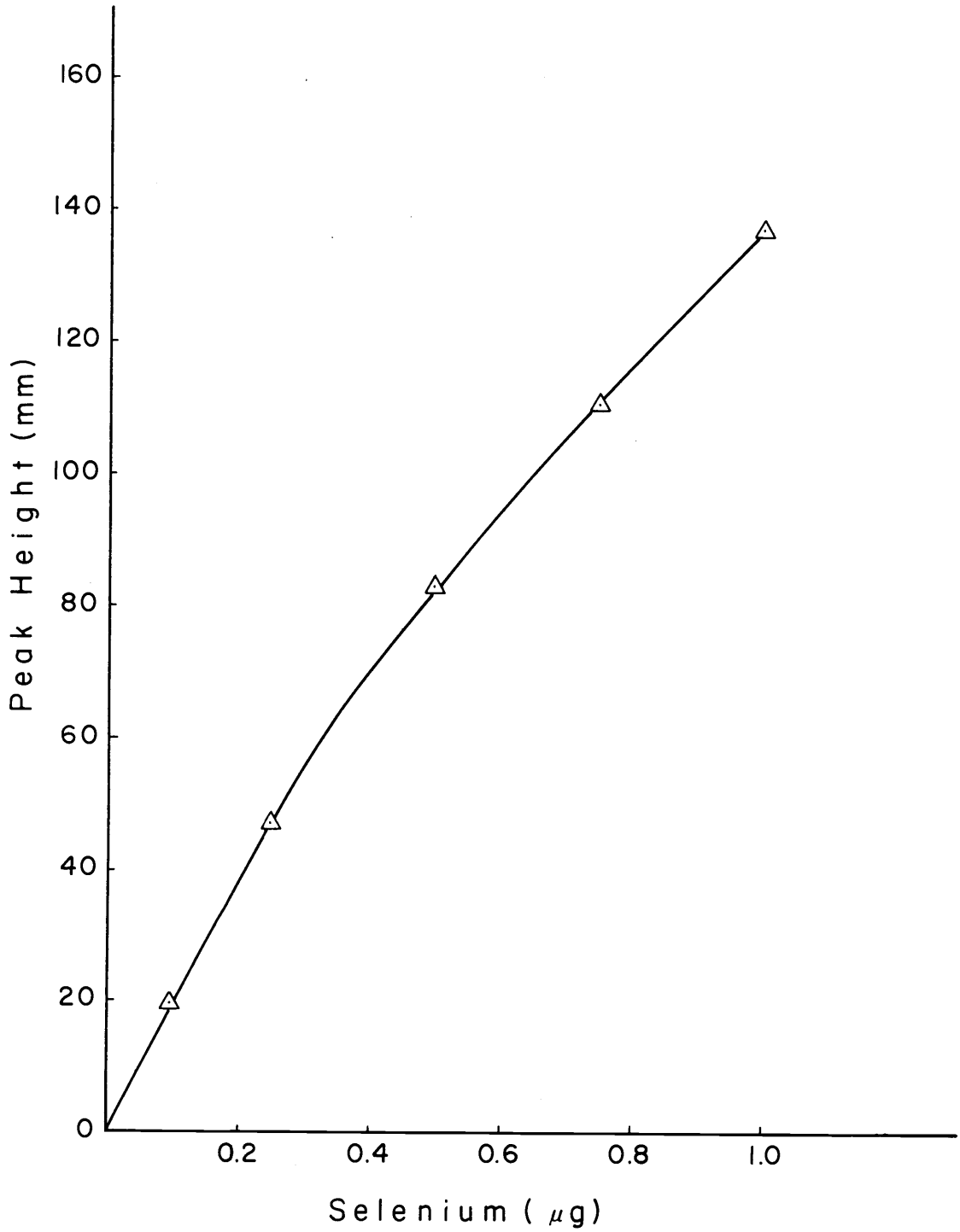

Figure 4. Calibration curve used to evaluate data in Table 4.

CONCLUSIONS

The complete digestion and reduction is the time consuming step and, therefore, a limiting factor. In the digestion of animal tissues in which insoluble materials are not formed, the procedure can be conducted in one vessel thereby eliminating the need for multiple transfers of sample. The complete method requires only one transfer, from the digestion flask to separatory funnel where the complexation is performed and the piaselenol extracted. The ease of handling and minimal number of steps are believed to outweigh the time consuming digestion.

The procedure should be applicable to any biological material which can be digested conveniently with this acid mixture. In the event that insoluble materials do form upon digestion they can easily be removed by filtration just prior to HCl refluxing. By collecting the filtrate in a second digestion flask and driving off the excess aqueous phase, the appropriate amount of 1 : 1 HCl can be added and the reduction step performed.

An advantage in this procedure is the use of the reduction step somewhat analogous to the arsenic coprecipitation technique as described by Allaway and Cary (1964). Both these methods would include selenium (VI) in the determination whether present naturally or formed in the oxidation step and thereby would give a more true

value for the total selenium present in the material. The inability to convert all selenium compounds to selenite in one oxidation step may account for the variation in results of the different techniques.

It should again be reiterated that the sensitivity of this method approaches that of the fluorometric methods using 2, 3-diaminonaphthalene as the complexing agent which, other than possibly neutron activation analysis, is the most sensitive method to date for selenium determination. The sensitivity could easily be improved further by using 4, 5 dichloro-o-phenylenediamine or the 4-nitro-o-phenylenediamine as suggested and used by Shimoishi and Toei (1970).

The instrumental parameters could be used to expand the output scale to increase the peak heights of the piaselenol. Even though the baseline noise is increased, an increased error can be tolerated when determining low total selenium content.

The relative standard deviations of each tissue analyzed showed a range from 2.3 to 4.8% with an overall value for the methodology of 3.5% which is comparable to other methods of selenium analysis in the same concentration range. The above results were obtained with representative samples of each tissue. In the analysis of whole tissues the biological variations can give selenium results which far exceed this 3.5% for replicate samples. It has been intimated in the literature that differences of over 50% have been obtained with

duplicate samples of certain tissues. The problem is then one of obtaining a representative sample.

It is believed that the method will be competitive with others in both speed and accuracy. This does not minimize the expertise needed for competent analysis, since this method and all others for selenium analysis require utmost care in the details of the analysis to obtain the desired results.

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Table A-1. Peak heights of ten consecutive injections of the same standard.

Order of injection	Peak height (mm)	Normalized values[a]
1	68.0	0.9659
2	67.0	0.9517
3	69.7	0.9901
4	71.0	1.0085
5	70.2	0.9972
6	69.7	0.9901
7	70.1	0.9957
8	70.0	0.9943
9	72.3	1.0270
10	75.5	1.0724

[a] Variance (ten injections); $s^2 = 0.001087$.

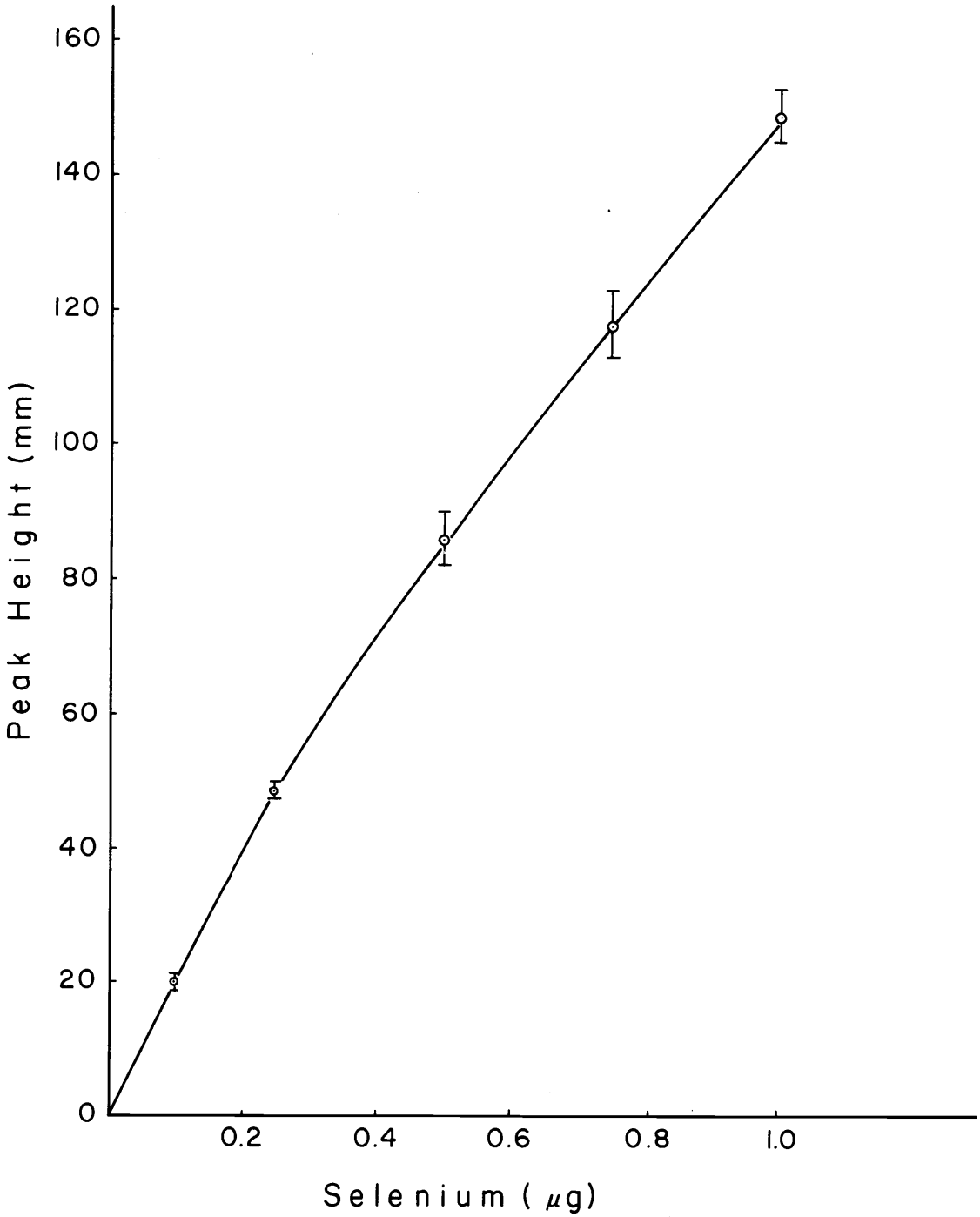

Figure A-1. Calibration curve using five replicate standards per data point.

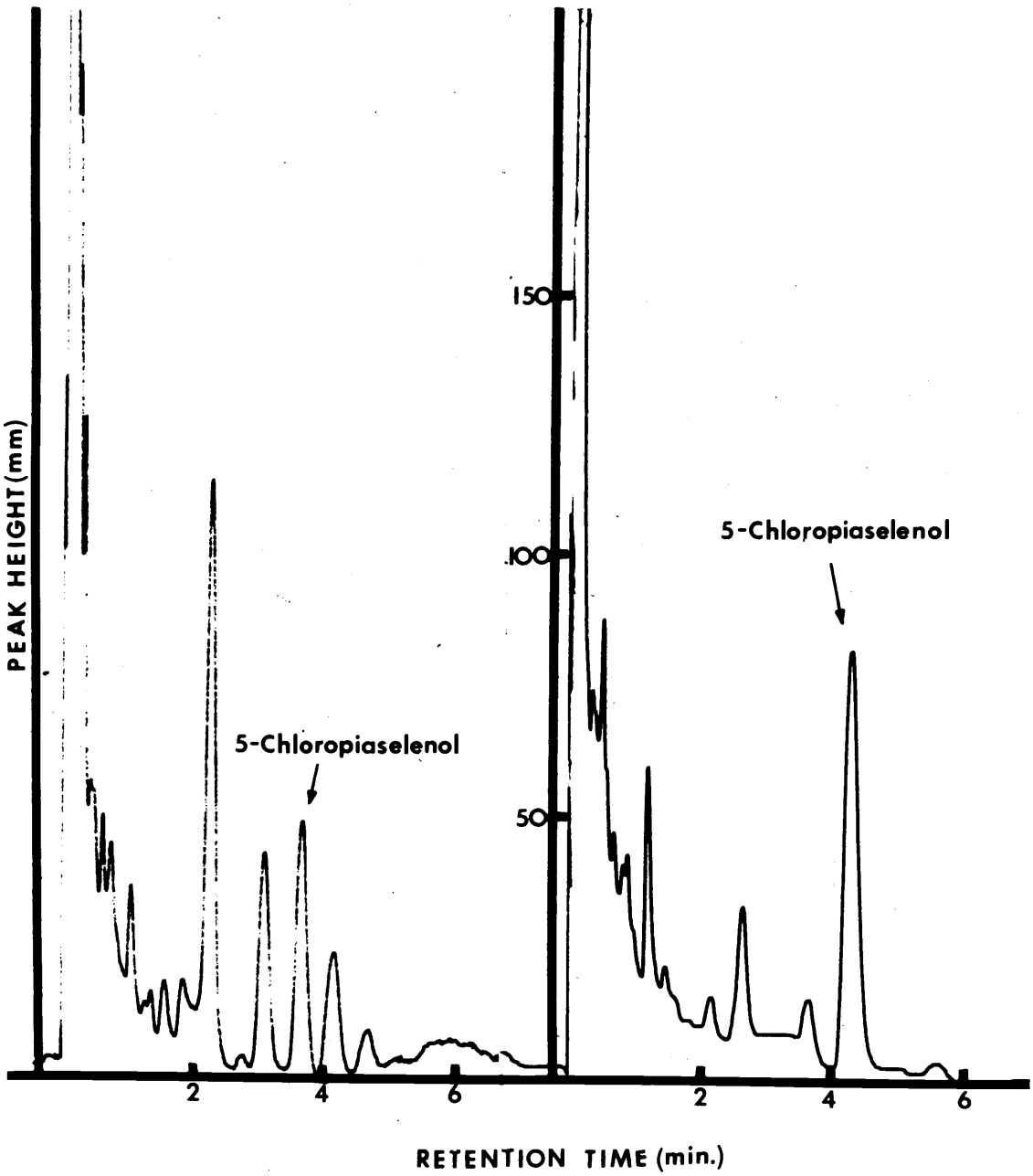

Figure A-2. Chromatogram from a human blood digest.

Figure A-3. Chromatogram from a lamb kidney digest.