

## Methods for the Isolation and Characterization of Constituents of Natural Products

### VIII. Gas-Liquid Chromatographic Resolution of Alcohol Ester, Amide, and Thioester Derivatives of Pyruvic Acid 2,6-Dinitrophenylhydrazone

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Parts I through VI of this series described the preparation, class separation, and thin-layer liquid-liquid partition chromatography of alcohol ester, amide, and thioester derivatives of pyruvic acid, 2,6-dinitrophenylhydrazone (9-13, 15). Part VII was devoted to purification of the derivatives with an anion exchange resin as a prerequisite to gas-chromatographic resolution of the derivatives (14). The present report is concerned with the resolution of homologous series of the derivatives by gas-liquid chromatography using both a flame ionization detector and an electron capture detector. The extreme sensitivity of the latter for polynitro compounds facilitates the detection of the derivatives in the millimicrogram range.

Resolution of phenylhydrazones (3), 2,4-dinitrophenylhydrazones of carbonyl compounds (4, 16), 3,5-dinitrobenzoates (4) and of *N*-2,4-dinitrophenyl amino acid esters (1, 2, 5, 6, 8) by gas chromatography using flame ionization detectors has already been described. Landowne and Lipsky (7) reported high orders of sensitivity for gas-liquid chromatography (GLC) of separated *N*-2,4-dinitrophenyl amino acid esters monitored by electron affinity spectrometry.

#### EXPERIMENTAL METHOD

*Samples and solvent.* Homologous series of esters of primary, secondary, and tertiary alcohols, amides of primary amines, and thio-

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ester derivatives of pyruvic acid 2,6-dinitrophenylhydrazone were obtained from stocks prepared and described by Schwartz and Brewington (9, 11, 13). Except for the tertiary alcohols, stock solutions of the first 10 members of each class were prepared in carbon disulfide and ethyl acetate to contain approximately 0.55  $\mu\text{M}/\text{ml}$  of each member of each class. The following tertiary alcohol derivatives were made up also in concentrations of approximately 0.55  $\mu\text{M}/\text{ml}$  each; *t*-butyl; *t*-amyl; 2-methyl-2-pentyl; 3-methyl-3-pentyl; 3-methyl-3-hexyl; 3-methyl-3-heptyl; 3-methyl-3-octyl.

In addition, a series of  $\text{C}_{1-15}$  primary alcohol derivatives was prepared to contain approximately 0.55  $\mu\text{M}/\text{ml}$  of each. Dilutions of the hydrazone solutions were made to establish lower limits of detection.

*Solvents.* Spectral grade of carbon disulfide was employed as the solvent for all of the hydrazone derivatives analyzed with the hydrogen flame detector and ACS grade ethyl acetate was used with the electron capture detector.

*Apparatus.*<sup>2</sup> An Aerograph model 204 gas chromatograph (Varian-Aerograph Co., Walnut Creek, Calif.) with a hydrogen flame ionization detector and an electron capture detector and a 0-1 mv Brown-Honeywell recorder were employed.

Samples were injected with a No. 701 Hamilton 10- $\mu\text{l}$  syringe. Syringes with teflon bushings were found to be unsatisfactory. The derivatives appeared to adhere to some extent to the teflon.

*Chromatographic conditions and procedure.* The GLC columns and instrument operating conditions for all analyses are presented in Table 1. From 1-3  $\mu\text{l}$  of the  $\text{CS}_2$  solutions were injected into the column when the hydrogen flame detector was employed and 1-5  $\mu\text{l}$  of ethyl acetate solutions with the electron capture detector.

Mixtures of each homologous series of the derivatives previously described, "Samples and Solvents," were separated by at least one of the sets of conditions described in Table 1.

To illustrate separation of higher molecular weight derivatives, a series of the pyruvic acid ester derivatives of the first 15 members of the primary alcohols was made up to contain approximately 0.55  $\mu\text{M}/\text{ml}$  each. This series was separated by conditions described in Table 1, Analysis 6.

<sup>2</sup> Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

TABLE I  
 CHROMATOGRAPHIC CONDITIONS FOR SEPARATION OF ESTERS, THIOESTERS, AND  
 AMIDE DERIVATIVES OF PYRUVIC ACID, 2,6-DINITROPHENYLHYDRAZONE

Analysis no.	Type of detector	Column <sup>a</sup> length (feet)	Mode of operation	Column packing	Temperature °C		Gas flow rate (ml/min)		
					Inj.	Det. Column	N <sub>2</sub>	H <sub>2</sub>	O <sub>2</sub>
1	Flame	6.5	Isothermal	3% J × R on 100/120 mesh gas chrom Q	250	235 230	84	45	84
2a		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	225	225 175-240 at 4°C/min	180	70	138
b		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	225	225 195-240 at 4°C/min	180	70	138
3	Electron capture	4	Isothermal	3% J × R on 100/120 mesh gas chrom Q	235	210 230	168	—	—
4a		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	220	210 200-235 at 2°C/min	168	—	—
b		4	Temperature program	3% J × R on 100/120 mesh gas chrom Q	220	210 180-235 at 4°C/min	168	—	—

Table 1 (Continued)

Analysis no.	Type of detector	Column <sup>a</sup> length (feet)	Mode of operation	Column packing	Temperature °C		Gas flow rate (ml/min)			
					Inj.	Det. Column	N <sub>2</sub>	H <sub>2</sub>	O <sub>2</sub>	
5a	Flame	1	Temperature program	5% SE 30 on 80/100 mesh AW-DMCS chrom W	220	240	150-220 at 4°C/min	50	40	80
b		1	Temperature program	5% SE 30 on 80/100 mesh AW-DMCS chrom W	220	240	135-200 at 4°C/min	50	40	80
6		2	Temperature program	5% SE 30 on 80/100 mesh AW-DMCS chrom W	215	240	150-235 at 4°C/min	80	52	84

<sup>a</sup> All columns were 1/8 inch o.d. stainless steel.

Response of the flame and EC detectors was compared by analyzing a series of secondary alcohol derivatives with each detector (see Table 1, Analyses 2b and 4b).

## RESULTS AND DISCUSSION

*Isothermal.* Table 2 shows the relative retention times of the first 10 members of primary and secondary alcohol and thiol derivatives separated isothermally by conditions described in Table 1 (Analyses 1 and 3).

TABLE 2  
RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6-DINITROPHENYLHYDRAZONE BY ISOTHERMAL (230°C) GLC ANALYSIS <sup>a</sup> ON 6.5 OR 4-FOOT J × R COLUMN

Carbon atoms	Primary alcohols		Secondary alcohols		Mercaptan	
	F	EC	F	EC	F	EC
1	0.04	0.05			0.09	0.10
2	0.06	0.06			0.11	0.11
3	0.07	0.08	0.05	0.06	0.14	0.14
4	0.09	0.10	0.07	0.07	0.18	0.19
5	0.12	0.13	0.09	0.09	0.24	0.24
6	0.16	0.17	0.12	0.11	0.32	0.32
7	0.20	0.21	0.16	0.15	0.42	0.44
8	0.27	0.28	0.20	0.20	0.56	0.58
9	0.35	0.36	0.27	0.26	0.78	0.75
10	0.47	0.47	0.34	0.33	1.00 <sup>b</sup>	1.00 <sup>c</sup>
11			0.47	0.44		
12			0.60	0.58		

<sup>a</sup> Conditions for analysis with the flame detector (F) are in Table 1, Analysis 1, and with the electron capture (EC) Analysis 3.

<sup>b</sup> Based on a retention time of 21.2 minutes.

<sup>c</sup> Based on a retention time of 15.6 minutes.

Although the actual retention time of the C<sub>10</sub> mercaptan derivative is longer by the conditions described in Analysis 1 than by Analysis 3 (21.2 vs 15.6 minutes), the relative retention times based upon the C<sub>10</sub> mercaptan derivative being 1.00 are almost identical.

It was apparent from the poor resolution of the first five members of each of the homologous series analyzed isothermally that temperature programming would be helpful in resolving these compounds.

TABLE 3  
RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6-DINITROPHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING (175–240°C AT 4°C/MIN) GLC ANALYSIS <sup>a</sup> ON 4-FOOT J × R COLUMN

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans
1	0.14		0.26
2	0.17		0.30
3	0.22	0.17	0.37
4	0.28	0.23	0.45
5	0.35	0.29	0.53
6	0.42	0.36	0.63
7	0.50	0.42	0.72
8	0.58	0.50	0.81
9	0.67	0.57	0.91
10	0.75	0.66	1.00 <sup>b</sup>
11		0.75	
12		0.83	

<sup>a</sup> For complete operating conditions see Table 1, Analysis 2a.

<sup>b</sup> Based on a retention time of 13.1 minutes.

*Temperature programming.* Table 3 presents relative retention times of derivatives separated by the conditions described in Table 1, Analysis 2a. Temperature programming from 175–240°C on the 4-foot J × R column gave better resolution of the early peaks and sharpened the later peaks. There was evidence on the inside of the flame detector cylinder of a considerable amount of column bleed at high temperature. Despite this, response was good and a suitable baseline could be maintained even without dual differential flame and columns. Later work showed, however, that column retention times changed with progressive column bleed.

Temperature programming the same J × R column but with an electron capture detector gave results similar to that obtained with the flame detector. These results (from conditions described in Table 1, Analysis 4a) are shown in Table 4. The differences in relative retention

times, Tables 3 and 4 (Analyses 2a and 4a) can be understood if one considers the different program temperature ranges and rates.

TABLE 4  
RELATIVE RETENTION TIMES OF ESTER AND THIOESTER DERIVATIVES OF PYRUVIC  
ACID 2,6-DINITROPHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING  
(200-235°C AT 2°C/MINUTE) GLC ANALYSIS<sup>a</sup> ON 4-FOOT J × R COLUMN

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans
1	0.13		0.22
2	0.14		0.24
3	0.18	0.15	0.30
4	0.22	0.19	0.37
5	0.28	0.22	0.43
6	0.33	0.27	0.53
7	0.41	0.33	0.62
8	0.48	0.39	0.71
9	0.57	0.47	0.83
10	0.66	0.55	1.00 <sup>b</sup>
11		0.64	
12		0.73	

<sup>a</sup> For complete operating conditions see Table 1, Analysis 4a.

<sup>b</sup> Based on a retention time of 16.1 minutes.

Table 5 shows the relative retention times of derivatives separated on a 1-foot SE 30 column temperature programmed from 150-220°C at 4°C per minute or 135-200°C (for tertiary alcohol derivatives). Complete operating conditions are described in Table 1, Analyses 5a and b. This short column could be operated at a lower temperature than the J × R column and thus primary alcohol derivatives with 15 carbons in the parent alcohol could be analyzed with good resolution before column bleed became a factor (see Fig. 1).

For some reason that has not been established the EC detector did not respond with the short SE 30 column.

Lower limits of detection are illustrated by Figs. 2 and 3. Although no attempts were made to relate peak size with concentration, it was obvious that one might quantitate the method. As can be seen from Fig. 2 there is no problem in analyzing solutions containing 0.055 μM/ml of the secondary alcohol derivatives with the hydrogen flame de-

TABLE 5  
RELATIVE RETENTION TIMES OF PRIMARY, SECONDARY AND TERTIARY ALCOHOL  
ESTER, AMIDE AND THIOESTER DERIVATIVES OF PYRUVIC ACID 2,6-DINITRO-  
PHENYLHYDRAZONE BY TEMPERATURE PROGRAMMING (150–220°C  
AT 4°C/min) ON 1-FOOT SE 30 COLUMN <sup>a</sup>

Carbon atoms	Primary alcohols	Secondary alcohols	Mercaptans	Amines
1	0.19		0.31	0.26
2	0.22		0.34	0.30
3	0.29	0.25	0.42	0.37
4	0.35	0.30	0.50	0.44
5	0.43	0.36	0.58	0.52
6	0.50	0.43	0.66	0.60
7	0.57	0.50	0.75	0.67
8	0.65	0.57	0.83	0.76
9	0.73	0.66	0.92	0.84
10	0.81	0.73	1.00 <sup>b</sup>	0.92
11		0.81		
12		0.90		
		Tertiary alcohols		
<i>t</i> -Butyl		0.45 <sup>c</sup>		
<i>t</i> -Amyl		0.55		
2-Methyl-2-pentyl		0.62		
3-Methyl-3-pentyl		0.62		
3-Methyl-3-hexyl		0.73		
3-Methyl-3-heptyl		0.80		
3-Methyl-3-octyl		1.00 <sup>d</sup>		

<sup>a</sup> For complete operating conditions see Table 1, Analyses 5a and 5b (for tertiary alcohols only).

<sup>b</sup> Based on retention time of 15.7 minutes.

<sup>c</sup> Temperature programming started at 135°C instead of 150°C.

<sup>d</sup> Based on a retention time of 12.8 minutes.

tector. Operating conditions for this analysis are shown in Table 1, Analysis 2b.

Even less material could be distinguished with the electron capture detector. Figure 3 shows the response from 5 µl of a solution containing 0.0055 µM/ml of each of the secondary alcohol derivatives. This represents only 10–15 µg of the derivative and 1–5 µg of the parent

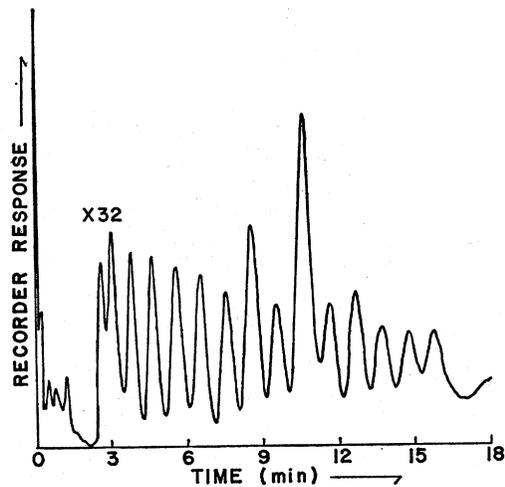


FIG. 1. The GLC pattern from  $1.1 \times 10^{-9}$  moles of each of  $C_{1-15}$  primary alcohol derivatives separated by temperature programming 150–240°C on a 2-foot SE 30 column and using a flame ionization detector.

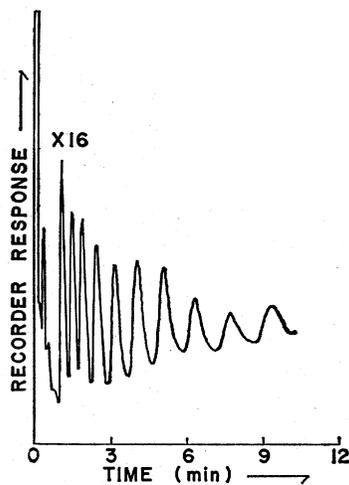


FIG. 2. The GLC pattern from  $1.1 \times 10^{-10}$  moles of each of  $C_{3-12}$  secondary alcohol derivatives separated by temperature programming 195–225°C on a 4-foot J × R column and using a flame ionization detector.

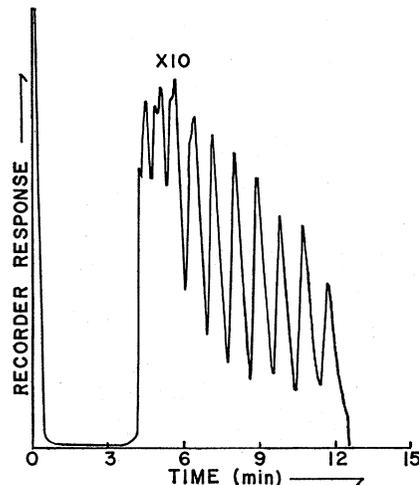


FIG. 3. The GLC pattern from  $2.5 \times 10^{-11}$  moles of each of  $C_{3-12}$  secondary alcohol derivatives separated by temperature programming 180–245°C on a 4-foot J  $\times$  R column and using an electron capture detector.

alcohol. Operating conditions to obtain the chromatogram in Fig. 3 are presented in Table 1, Analysis 4b.

Initially, glass liners were employed in the injection block but it was found that when the injector port was kept clean, glass liners were not essential. Care was taken also that the flame tips in particular and the detector in general were kept clean.

Changes in retention times were found to occur with extensive use of these columns. Although the retention times did change, conditions could be altered to maintain the good resolution obtained with the new column. It is very possible that silicone oil columns less inclined to bleed may eliminate or reduce this problem.

It would be necessary, of course, for identification purposes, to run standard solutions during the same sequence that unknowns are being analyzed. Since these derivatives are stable and easily maintained, this presents no problem.

*Double peaks.* When conditions were employed to increase separation of the first three members of each homologous series, each was

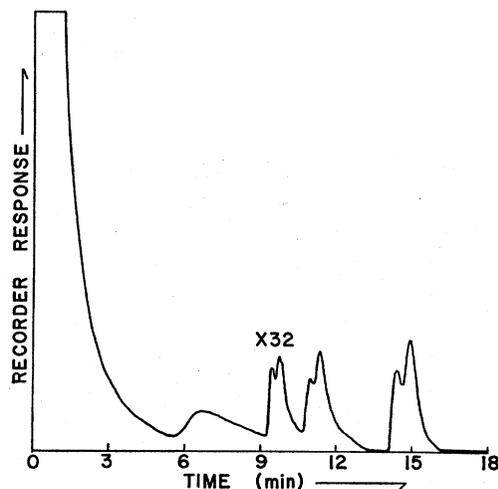


FIG. 4. The GLC pattern showing separation of each of the first three primary alcohol derivatives ( $C_{1-3}$ ) each into two peaks isothermally at  $200^{\circ}\text{C}$  on a 6.5-foot  $J \times R$  column and using a hydrogen flame detector.

found to separate into two peaks. Figure 4 illustrates this condition. With the 6.5-foot  $J \times R$  column described in Table 1, Analysis 1 operated at a column temperature of  $200^{\circ}\text{C}$ , the first three members of primary alcohol derivatives shown in Fig. 4 separate clearly into two peaks. These doublet peaks were merged into single peaks by increasing the column temperature to  $230^{\circ}\text{C}$ .

With the small sample employed to determine lower limits of detection with the electron capture detector, the doublet peaks also were apparent. This can be seen in the chromatogram (Fig. 3) of the homologous series of millimicrogram levels of secondary alcohol derivatives monitored with the EC detector.

The authors feel that each of the single peaks represented in the figures could be resolved into two peaks on the  $J \times R$  column with an appropriate column temperature. To simplify interpretation of the results, conditions were employed to yield one peak from each member of each homologous series.

It is possible that the doublet peaks represent isomeric forms of the derivatives present in the original crystals. However, we have been unable to obtain any evidence for isomers by thin-layer or

column adsorption chromatography on silica gel or on magnesium oxide, or by thin-layer and column liquid-liquid partition chromatograms. The appearance of doublet peaks has not been apparent by gas-liquid chromatography using 6 foot  $\times$  0.25-inch J  $\times$  R columns in some other instruments (Research Specialties and Barber-Coleman) under conditions which produce the double peaks on the Aerograph 204.

The question of whether the crystalline derivatives used in this study contain 2 isomers or whether the isomers are produced during the gas chromatographic operation has not been resolved as yet. Additional experiments are underway to settle this question. Either way, the problem is not serious from an analytical standpoint since conditions can be chosen during gas chromatography to eliminate the appearance of the double peaks, and no evidence for double spots or bands has been encountered during thin-layer or column chromatography.

#### SUMMARY

Gas-liquid chromatographic techniques have been employed for the resolution of homologous series of primary, secondary, and tertiary alcohol ester- primary amine amide- and thioester derivatives of pyruvic acid 2,6-dinitrophenylhydrazones. The derivatives can be separated isothermally or by temperature programming on 6.5 or 4-foot J  $\times$  R or on a 1-foot SE 30 column and can be detected by flame or electron capture detectors. The latter permits the detection of from 10-15  $\mu\text{g}$  of a derivative which is equivalent to about 1-5  $\mu\text{g}$  of the parent compound.

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