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Note

Gas-liquid chromatographic analysis of 2,4-dinitrophenylhydrazones of keto acid methyl esters

For gas–liquid chromatographic (GLC) analysis, the keto acids associated with amino acid pool and energy production have to be converted to various volatile derivatives, such as methyl esters[1–3], methyl oximes of methyl or trimethylsilyl esters[4,5], trimethylsilyl oximes of trimethylsilyl esters[6,7], or trimethylsilyl derivatives of quinoxalones[8].

The 2,4-dinitrophenylhydrazones of keto acids, which can be used successfully for the isolation of these acids[9], have not previously been analysed by GLC. However, as we have obtained reliable results by analysing the 2,4-dinitrophenylhydrazones of carbonyl compounds by GLC[10], we have, in the present work, carried out experiments also on keto acid hydrazones after converting them to methyl esters.

Experimental

The 2,4-dinitrophenylhydrazones were prepared in the usual manner from 2,4-dinitrophenylhydrazine (guaranteed reagent grade, E. Merck A.G.)[10], twice recrystallized from carbonyl-free ethanol (Oy Alko Ab, AaS), and from the following keto acids: glyoxylic, pyruvic, 2-oxobutyric, 2-oxo-3-methylbutyric, 2-oxo-3-methylvaleric, 2-oxo-4-methylvaleric, oxaloacetic and 2-oxoglutaric acid. The 2,4-dinitrophenylhydrazones of keto acids were converted into their methyl esters with a mixture of methanol (guaranteed reagent grade, E. Merck A.G.) and hydrochloric acid (guaranteed reagent grade, E. Merck A.G.)[11]. The precipitated keto acid methyl ester 2,4-dinitrophenylhydrazones were washed with 2 *N* hydrochloric acid and water and dissolved in ethyl acetate (guaranteed reagent grade, E. Merck A.G.) for GLC analysis.

The gas chromatograph used was a Varian Aerograph Model 2100-20 equipped with U-shaped glass columns 1.8 m long and 2 mm I.D. and with two flame ionization detectors (FID) and a ^{63}Ni (8 mCi) electron capture detector (ECD). The column filling was 4 % (w/w) SE-30 methyl silicone gum (for gas chromatography, Varian Aerograph) on Chromosorb W (80–100 mesh, acid washed and trimethylchlorosilylated, Varian Aerograph). The columns were conditioned as described earlier[10].

When the electron capture detector was used, the temperature of the column was 220° and that of the detector foil 300°. When the hydrogen flame ionization detectors were used, the temperature of the columns was 220° during the first 17 min and was then raised to 260° at the rate of 4°/min. In all analyses, the temperature of the injection block was 240° while the flow-rate of nitrogen through the columns was 30 ml/min.

Results and discussion

The gas chromatograms of 2,4-dinitrophenylhydrazones of the methyl esters

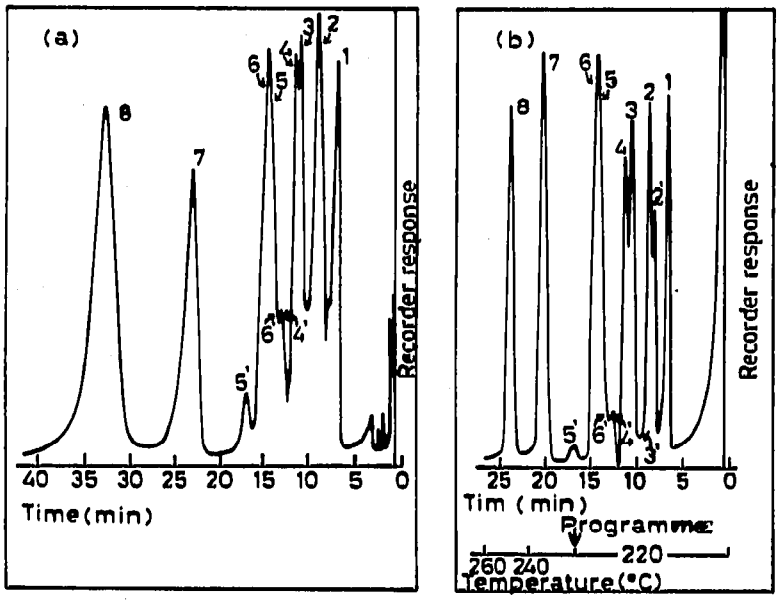

Fig. 1. Gas–liquid chromatogram of a mixture of 2,4-dinitrophenylhydrazones of keto acid methyl esters resolved on a 4% SE-30 column. 1 = Glyoxylic; 2 = pyruvic; 3 = 2-oxobutyric; 4 = 2-oxo-3-methylbutyric; 5 = 2-oxo-3-methylvaleric; 6 = 2-oxo-4-methylvaleric; 7 = oxaloacetic; 8 = 2-oxoglutaric acid. The isomeric forms of the respective compounds are indicated by 2′, 3′, 4′, 5′ and 6′. (a) Analysis on a gas chromatograph equipped with a ^{63}Ni electron capture detector. Constant temperature (220°). Injected sample: 1.0 μl of hexane containing 0.5–5 ng amounts of each of the derivatives. Attenuation 8×10^{-10} A. (b) Analysis on a gas chromatograph equipped with a hydrogen flame ionization detector. Constant temperature (220°) during the first 17 min, then the temperature was raised from 220° to 260° at the rate of 4°/min. Injected sample: 1.0 μl of ethyl acetate containing 100–500 ng amounts of each of the derivatives.

TABLE I

RELATIVE RETENTION TIMES OF 2,4-DINITROPHENYLHYDRAZONES OF KETO ACID METHYL ESTERS

The retention time of the derivative of 2-oxobutyric acid was taken as unity. Determinations were carried out on a 4% SE-30 column (a) at 220° (ECD) and (b) by keeping the temperature at 220° for 17 min and then continuing with a linear temperature programme to 260° at the rate of 4°/min (FID)

Keto acid	*Relative retention time*	
	(*a*)	(*b*)
Glyoxylic acid	0.63	0.63
Pyruvic acid	0.82	0.82
2-Oxobutyric acid	1.00	1.00
2-Oxo-3-methylbutyric acid	1.06	1.06
2-Oxo-3-methylvaleric acid	1.31	1.31
2-Oxo-4-methylvaleric acid	1.32	1.32
Oxaloacetic acid	2.04	1.84
2-Oxoglutaric acid	2.91	2.13

of the metabolically important keto acids mentioned above are shown in Fig. 1. The chromatogram recorded for isothermal analysis with the electron capture detector is shown in Fig. 1a and that recorded in a combined isothermal and temperature-programmed analysis with a hydrogen flame ionization detector is shown in Fig. 1b. The retention times of the keto acid derivatives studied relative to the retention time of the derivative of 2-oxobutyric acid are summarized in Table I.

The results show that under the described conditions the keto acid derivatives are separated fairly well on the SE-30 column with the exception of two critical pairs of acids. Of these, the derivatives of 2-oxobutyric acid and 2-oxo-3-methylbutyric acid are only partially separated, whereas the derivatives of isomeric 2-oxo-3-methylvaleric acid and 2-oxo-4-methylvaleric acid were not separated from each other at all. When the temperature was lowered to 180°, the separation of the first pair of acids improved to 60% but the latter pair of acids gave only a slightly asymmetric peak. At this temperature, however, the retention times become too long, the duration of analysis being nearly 2 h. Considering the derivatives of all the keto acids mentioned above, the best results were obtained with a combined isothermal and temperature-programmed elution under the conditions described in Fig. 1b.

In the chromatograms of the 2,4-dinitrophenylhydrazones of keto acid methyl esters, the formation of a double peak is observed, which is characteristic of derivatives of keto monocarboxylic acids but not of the derivatives of keto dicarboxylic acids. In Figs. 1a and 1b, "secondary peaks" can be seen between the main peaks 1 and 2, 4 and 5, and 6 and 7. These smaller peaks are indicated as 2', 3', 4', 5' and 6'. Experiments with individual keto acid derivatives show which of the derivatives cause the double peaks, whether the secondary peak comes before or after the main peak. The appearance of similar double peaks caused by isomeric forms of 2,4-dinitrophenylhydrazone derivatives of carbonyl compounds have previously been observed when they were analysed by GLC[10]. The formation of a double peak was found to depend particularly on the solvent used.

The method developed during this study for the GLC analysis of 2,4-dinitrophenylhydrazones of keto acid methyl esters was very successful in comparison, for instance, with the GLC analysis of the methyl esters liberated from the respective hydrazones by ozonization[3]. Contrary to our method, that method resulted in diverse by-products formed in various amounts and, furthermore, it cannot be used for the determination of keto dicarboxylic acids.

The results of the GLC analyses of the 2,4-dinitrophenylhydrazones of the keto acid methyl esters studied show that amounts as small as 10 ng can be determined with a hydrogen flame ionization detector. Hence the sensitivity is of the same order of magnitude as that obtained with trimethylsilyl derivatives[7]. When the electron capture detector was used, the sensitivity for the 2,4-dinitrophenylhydrazones of keto monocarboxylic acid methyl esters was 100 times greater and for the derivatives of keto dicarboxylic acids 20 times greater than when a hydrogen flame ionization detector was used.

The linear response was broad, *viz.*, 10–1000 ng with a hydrogen flame ionization detector, but very narrow when an electron capture detector was used. However, the sensitivity and the selectivity of the electron capture detector for the derivatives

of the above dinitrophenylhydrazones are noteworthy and the method is therefore suitable for the analysis of very small amounts of keto acids in biological material.

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1 F. L. ESTES AND R. C. BACHMANN, *Anal. Chem.*, 38 (1966) 1178.
2 M. MAKITA AND W. W. WELLS, *Anal. Biochem.*, 5 (1963) 523.
3 P. RONKAINEN AND S. BRUMMER, *J. Chromatogr.*, 28 (1967) 259.
4 Y. ISHITOYA, C. ITOH, N. OSAWA, I. HASHIMOTO, T. IWANAGA AND T. NAMBARA, *Clin. Chim. Acta*, 27 (1970) 233.
5 M. G. HORNING, E. A. BOUCHER, A. M. MOSS AND E. C. HORNING, *Anal. Lett.*, 1 (1968) 713.
6 Z. HORII, M. MAKITA AND Y. TAMURA, *Chem. Ind. (London)*, 34 (1965) 1494.
7 H. ROSENQVIST, H. KALLIO AND V. NURMIKKO, *Anal. Biochem.*, 46 (1972) 224.
8 N. E. HOFFMAN AND T. A. KILLINGER, *Anal. Chem.*, 41 (1969) 162.
9 C. KAWANO, H. KATSUKI, T. YOSHIDA AND S. TANAKA, *Anal. Biochem.*, 3 (1962) 361.
10 H. KALLIO, R. LINKO AND J. KAITARANTA, *J. Chromatogr.*, 65 (1972) 355.
11 W. STOFFEL, F. CHU AND E. H. AHRENS, *Anal. Chem.*, 31 (1959) 307.

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