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GAS CHROMATOGRAPHIC ANALYSIS OF α-KETO ACIDS IN AQUEOUS SOLUTION AS THE O-(2,3,4,5,6-PENTAFLUOROBENZYL)OXIMES OF THEIR METHYL ESTERS

KEIKO KOBAYASHI*, ETSUO FUKUI, MICHIRU TANAKA and SATOSHI KAWAI
Gifu College of Pharmacy, Mitahora Higashi, Gifu (Japan)
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SUMMARY

As an extension of previous work on carbonyl compounds, a method is described for the gas chromatographic analysis of α-keto acids in aqueous solution. The keto acids were derivatized with pentafluorobenzyloxylamine to yield the oximes. After extraction with ethyl acetate from acidified, salt-saturated solution, these compounds were esterified with diazomethane and separated by gas chromatography.

INTRODUCTION

An elevated excretion of α-keto acids has proved to be of great value as a biochemical marker of hereditary metabolic diseases such as maple syrup urine disease and phenylketonuria. For gas chromatographic (GC) analysis, α-keto acids have to be converted into various volatile and/or stable derivatives, such as methyl esters[1], methyloximes of trimethylsilyl esters[2], trimethylsilyloximes of trimethylsilyl esters[3], O-trimethylsilylquinoxalinol derivatives[4–8] and 2,4-dinitrophenylhydrazone methyl esters[9]. The 2,4-dinitrophenylhydrazones of α-keto acids have been used successfully for the isolation of these acids. Kallio and Linko[9] carried out the GC of the α-keto acid hydrazones after converting them into methyl esters. Recently, we reported that pentafluorophenylhydrazones[10] or O-pentafluorobenzyloximes (O-PFBO)[11] of carbonyl compounds were much more volatile than the corresponding 2,4-dinitrophenylhydrazones, and therefore their GC separation could be performed at much lower temperatures. As an extension of our work on carbonyl compounds, this paper describes a method for the separation and quantification of α-keto acids in aqueous solution by GC as the O-PFBO derivatives of their methyl esters.

EXPERIMENTAL

Reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine (pentafluorobenzyloxylamine, PFBOA) hydrochloride was synthesized from pentafluorobenzyl bromide (Aldrich,

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Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan)[11]. α-Keto acids (pyruvate, α-ketobutyric acid, α-ketovaleric acid, α-ketoisovaleric acid, α-ketocaproic acid, α-ketoisocaproic acid, α-keto-β-methyl-*n*-valeric acid and α-ketooctanoic acid) were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample solutions were aqueous solutions containing 0.5 μmol/ml of each α-keto acid. The internal standard (IS) was a 0.5 μmol/ml solution of *p*-xylylene dichloride in ethyl acetate.

Apparatus and conditions

A Shimadzu Model GC-4APF gas chromatograph equipped with a hydrogen flame-ionization detector (FID) and a GC-4APE gas chromatograph equipped with a 10-mCi Ni-63 electron-capture detector (ECD) were used. Separations were carried out by using a glass column, 2.0 m × 3 mm I.D., packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS). The column temperature was 130°C, the detector temperature 150°C, the injection temperature 150°C and the chart speed 0.25 cm/min.

Standard procedure

To a 1.0-ml aliquot of sample solution containing α-keto acids in a 10-ml centrifuge tube was added 0.5 ml of PFBOA solution (1.0 mg/ml; *ca.* $4.0 \cdot 10^{-6}$ *M*). The mixture was shaken well and allowed to stand for 30 min at room temperature. After saturation with sodium chloride and acidification with 1 drop of 18 *N* sulphuric acid, the O-PFBO derivatives of the α-keto acids were extracted with 1.0 ml of ethyl acetate containing *p*-xylylene dichloride (0.5 μmol/ml) as internal standard. Excess of sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle. After evaporation of the ethyl acetate extract in another small vessel, the oximes were converted into their methyl esters with freshly prepared diazomethane in diethyl ether. The reaction mixture was evaporated to dryness again and the residue of methyl esters was dissolved in 0.3 ml of ethyl acetate for GC analysis. Quantitation was carried out using calibration graphs obtained from known amounts of α-keto acids.

RESULTS AND DISCUSSION

The retention times of the O-PFBO derivatives of eight α-keto acid methyl esters relative to that of pyruvate obtained on different columns are given in Table I. In the chromatograms of the O-PFBO derivatives of α-keto acid methyl esters, the formation of a double peak was observed, corresponding to *syn* and *anti* isomers. The appearance of similar double peaks caused by isomeric forms of O-PFBO derivatives of carbonyl compounds has previously been observed on gas chromatograms[11]. This stereoisomerism seemed to make qualitative and quantitative work with α-keto acids difficult and has, in fact, been indicated as a disadvantage of hydrazones or oximes derivatives for the GC of carbonyl compounds. However, PFBOA has the following merits as a derivatization agent for their GC. The reaction of carbonyl compounds with PFBOA proceeded readily in weakly acidic media (pH 2–5) at room temperature to yield compounds extractable from aqueous solution with organic solvents, and the complete removal of unreacted reagent was

TABLE I

RELATIVE RETENTION TIMES OF O-PENTAFLUOROBENZYLOXIMES OF α-KETO ACID METHYL ESTERS

α-Keto acid	*Stationary phase and column temperature*			
	3% XE-60 (130°C)	*1.5% SE-30 (110°C)*	*1.5% OV-17 (100°C)*	*2% OV-1 (120°C)*
Pyruvic acid	0.73, 1.00* (1:17.7)	0.72, 1.00	0.72, 1.00	0.75, 1.00
α-Ketobutyric acid	0.95, 1.15 (1:4.3)	1.05, 1.32	0.99, 1.27	1.05, 1.26
α-Ketovaleric acid	1.32, 1.54 (1:1.5)	1.52, 1.87	1.46, 1.83	1.53, 1.91
α-Ketoisovaleric acid	1.02, 1.20 (6.5:1)	1.25, 1.52	1.06, 1.39	1.17, 1.40
α-Ketocaproic acid	1.95, 2.27 (1:2.3)	2.51, 3.06	2.42, 3.05	2.30, 2.79
α-Ketoisocaproic acid	1.51, 1.73 (1:1.4)	1.97, 2.39	1.69, 2.14	1.78, 2.15
α-Keto-β-methyl-*n*-valeric acid	1.44, 1.56 (8.8:1)	1.83, 2.13	1.63, 1.99	1.74, 2.10
α-Ketooctanoic acid	3.76, 4.02 (1:6.8)	6.03, 6.94	6.36, 7.65	5.99, 7.05

* The retention time of pyruvate was taken as unity; all α-keto acids showed double peaks corresponding to *syn* and *anti* isomers resulting from condensation reactions with PFBOA. Values in parentheses show the ratio of the areas of the two peaks on the chromatogram.

easily achieved. The resulting derivatives were highly stable in organic solvents, much more volatile than other derivatives, and therefore their GC separation could be carried out at much lower temperatures. Also, the O-PFBO derivatives were extremely sensitive to the electron-capture detector.

O-Trimethylsilyl-quinoxalinols are very useful derivatives for the GC of α-keto acids because of the absence of stereoisomerism[5–8]. They are eluted as a single peak on the chromatogram. However, with regard to rapid reaction, volatility of the derivatives, sensitivity to the electron-capture detector and simplicity of procedure, PFBOA is a much better derivatization agent. In addition, the double peaks consisted of a main peak and a smaller peak (shown in parentheses in Table I) and under constant reaction conditions the main peak was sufficiently stable for determination. All measurements were performed using main peaks. The smaller peak usually came before the main peak, but the ratio of the peak area of the second one (with the longer retention time) to that of the first became smaller with increasing bulk of the R group in the α-keto acid (R-CO-COOH), and with α-ketoisovaleric acid and α-keto-β-methyl-*n*-valeric acid the ratio was reversed, presumably owing to steric hindrance by a methyl group in position 3. The above phenomena suggest that the first of the double peaks is the *syn*- form.

Typical GC separations of some α-keto acids as their O-PFBO methyl derivatives are illustrated in Fig. 1, in which the smaller "secondary peaks" of each α-keto acid are indicated as 1′, 2′ and 3′. The O-PFBO methyl derivatives were about 1000 times more sensitive to the ECD than to the FID. A stable response was

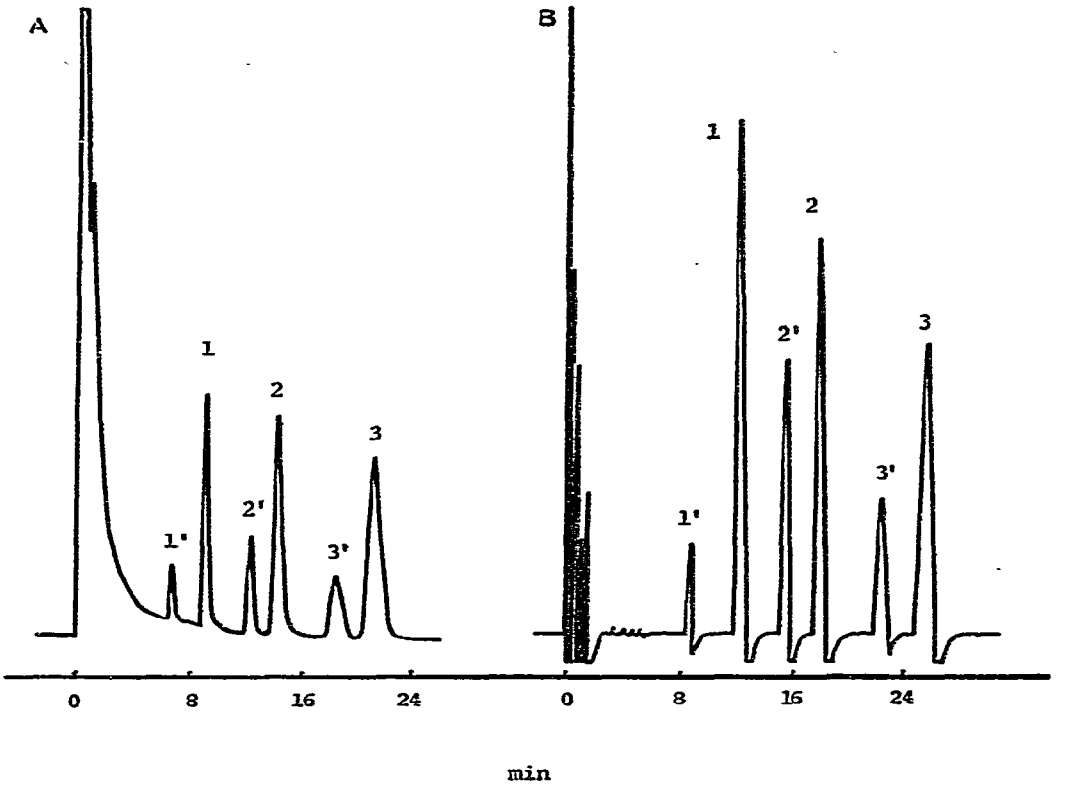

Fig. 1. Gas chromatograms of a mixture of three α-keto acids as their pentafluorobenzyloxime methyl esters on a 3% GE XE-60, 2.0-m glass column, 130°C. 1 = Pyruvic acid; 2 = α-ketovaleric acid; 3 = α-ketocaproic acid. The secondary peaks of the compounds are indicated by 1′, 2′ and 3′, respectively. (A) Analysis with an FID; 0.5 μg of each α-keto acid was injected. (B) Analysis with an ECD; 0.5 ng of each α-keto acid was injected.

observed on the chromatogram even for the injection of 10 pg of α-keto acids as their O-PFBO derivatives.

A series of preliminary investigations was carried out in order to find suitable conditions for reaction and extraction. The formation of the O-PFBO derivatives in aqueous solution was easily achieved with low concentrations of PFBOA. The reagent concentration was made about four times greater than those of the α-keto acids. Fig. 2 shows the effect of variation of pH on the extent of the condensation reaction with 1.0 ml of 0.5 μmol/ml of α-ketobutyric acid according to the procedure described under Experimental. The measured values were constant at pH 2–5 (see Fig. 2).

The effects of variations in the reaction temperature and reaction period on the extent of the condensation reaction were investigated. Fig. 3 shows the results with 1.0 ml of 0.5 μmol/ml of pyruvate. As can be seen, the condensation reaction was complete in 30 min at room temperature, after which the measured values were constant for up to 120 min. The reaction period for α-keto acids was therefore fixed at 30 min at room temperature in order to obtain constant extents of reaction. Ethyl

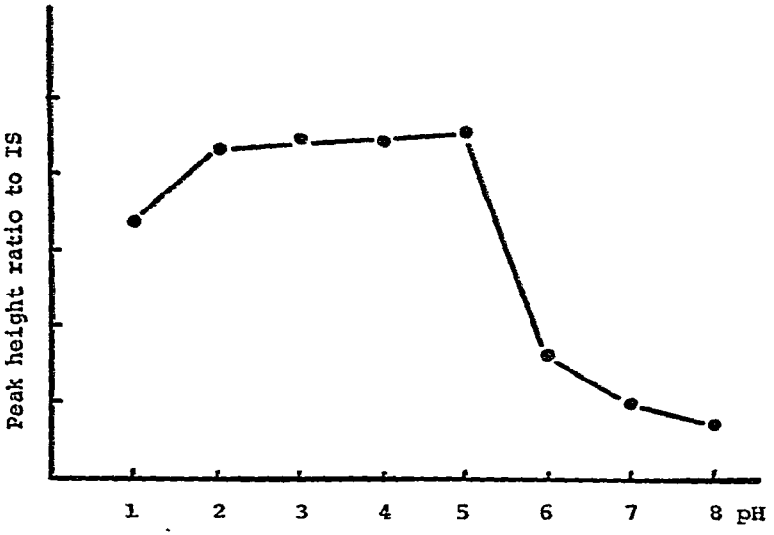

Fig. 2. Effect of pH on the condensation reaction of α-ketobutyric acid with PFBOA in aqueous solution.

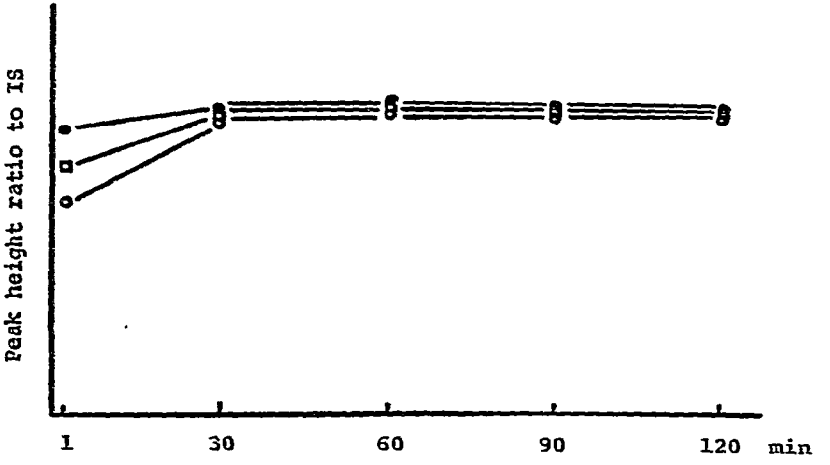

Fig. 3. Effects of variations in the reaction temperature and reaction period on the extent of the condensation reaction of pyruvate with PFBOA in aqueous solution. ○, 0°C; □, room temperature; ●, 60°C.

acetate was a suitable solvent for the extraction of the oximes. Salting-out improved the extent of extraction.

The effect of pH of the solution on the extent of extraction was examined. With the O-PFBO derivative of pyruvate, the extent of extraction increased with increasing acidity of the medium. In addition, in order to prevent an excess of PFBOA from being extracted, extraction was carried out in acidic media. A sample solution containing individual α-keto acids was measured according to the procedure described and linear relative response graphs passing through the origin were obtained with all of the α-keto acids studied for concentrations in the range 0.2–1.0 μmole in 1.0 ml of aqueous solution (Fig. 4).

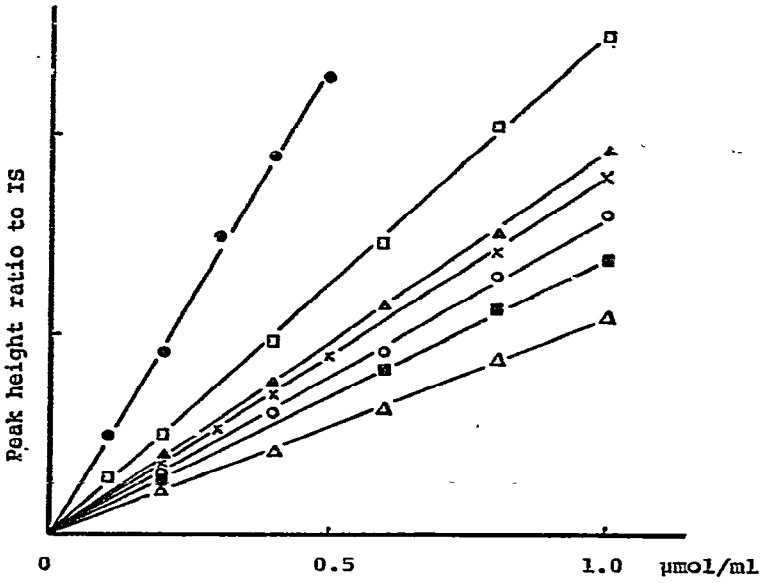

Fig. 4. Calibration graphs for some α-keto acids. ■, Pyruvic acid; △, α-ketobutyric acid; ●, α-ketovaleric acid; □, α-ketoisovaleric acid; ▲, α-ketocaproic acid; ×, α-ketoisocaproic acid; ○, α-keto-β-methyl-*n*-valeric acid. Conditions: 3% XE-60, 2.0-m glass column, FID. *p*-Xylylene dichloride was used as an internal standard.

On an identical sample solution containing 0.5 μmol/ml of α-keto acid in aqueous solution, the coefficient of variation after repeated derivatizations was 4.5% for pyruvate ($n = 7$) and 1.9% for α-ketobutyric acid ($n = 5$).

From the above results, it is concluded that PFBOA is an excellent derivatization agent for the GC determination of α-keto acids in aqueous solution and therefore the method should be of use for the determination of very small amounts of α-keto acids in biological fluids.

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