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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PYRUVIC ACID AND α-KETOGLUTARIC ACID IN SERUM

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## SUMMARY

A high-performance liquid chromatographic determination of pyruvic acid and α-ketoglutaric acid in serum is described which is based on the formation of 2,4-dinitrophenylhydrazones. The *syn–anti* isomerization of the 2,4-dinitrophenylhydrazone of pyruvic acid is also discussed.

## INTRODUCTION

Pyruvic acid and α-ketoglutaric acid exist in serum, as metabolites of sugars, lipids and amino acids. Changes in their concentrations indicate abnormal disturbance of the biological system. Many methods[1–5] have been reported for the determination of α-keto acids in biological samples. A colorimetric method using 2,4-dinitrophenylhydrazine (DNP) has been most widely used. Although DNP reacts with α-keto acids to form the corresponding hydrazone (DNPH) under mild conditions, this reaction is not specific for α-keto acids.

The purpose of the present study was to establish a specific and stable method for the determination of pyruvic acid and α-ketoglutaric acid by combining the above reaction with high-performance liquid chromatography (HPLC).

## EXPERIMENTAL

### *Apparatus and reagents*

The apparatus used was a DuPont Model 840 liquid chromatograph equipped with a UV absorption detector (254 nm). The separation was carried out with a column (50 cm × 2.1 mm I.D.) of Zipax Permaphase AAX (particle size, 30–50 μm) purchased from Shimadzu Seisakusho, Kyoto, Japan.

Sodium pyruvate, α-ketoglutaric acid and DNP were obtained from Wako, Osaka, Japan, and trimellitic acid from Tokyo Organic Chemicals, Tokyo, Japan. Ethyl acetate and methanol were used after distillation. The other reagents and organic solvents used were reagent grade.

*Procedure*

To 0.5 ml of serum, 3.5 ml of water, 0.5 ml of 5% sodium tungstate and 0.5 ml of 0.33 *N* sulphuric acid were added, mixed vigorously and centrifuged (3000 rpm, 5 min). To 3.0 ml of the supernatant, 2.0 ml of internal standard solution (2.5 $\mu$g of trimellitic acid in 1 ml of water) and 5 mg of DNP in 5 ml of 1 *N* hydrochloric acid were added. The mixture was allowed to stand for 30 min at room temperature, and then 5 ml of 0.6 *M* sodium carbonate were added. The mixture was washed three times with chloroform (5 ml each time). After adding 4 g of sodium chloride and 10 ml of 0.5 *N* hydrochloric acid, the hydrazones produced were extracted with 10 ml of ethyl acetate. The organic phase was dried over sodium sulphate and the ethyl acetate was evaporated by use of a rotatory evaporator. The residue was dissolved in a few drops of methanol. 10 $\mu$l of this solution were subjected to HPLC.

## RESULTS AND DISCUSSION

2,4-Dinitrophenylhydrazine reacts not only with keto acids but also with various carbonyl compounds. Only keto acids, however, give the DNPH derivative which has one or more carboxyl groups. This derivatization of keto acids results in a large increase in their affinity for anion-exchange columns. Therefore, an anion-exchange chromatographic system was considered to be convenient for the determination of keto acids in various biological samples which contain many kinds of carboxylic acids.

The chromatographic behaviour of the DNPH derivatives of pyruvic acid and

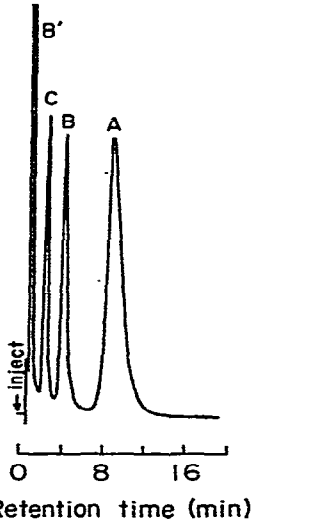



Fig. 1. Liquid chromatogram of a standard mixture of the DNPH derivatives of pyruvic acid and $\alpha$-ketoglutaric acid, and trimellitic acid (internal standard). Operating conditions: column, 50-cm Zipax Permaphase AAX (particle size, 30–50 $\mu$m); mobile phase, 11.1 g of $KH_2PO_4$ + 0.9 g of $K_2HPO_4$ per l of water; column temperature, 55°; flow-rate, 1.2 ml/min (pressure, 50 kg/cm$^2$); detector, UV photometer (254 nm). Peaks: A = DNPH–$\alpha$-ketoglutaric acid; B, B' = DNPH–pyruvic acid; C = trimellitic acid.

α-ketoglutaric acid, and of the internal standard trimellitic acid, was investigated on a 50-cm column of Zipax Permaphase AAX in aqueous phosphate solution. Fig. 1 shows a typical chromatogram obtained from a standard mixture of the DNPH derivative and trimellitic acid. DNPH–pyruvic acid exhibited two peaks, B and B′, which were presumed to arise from the *syn–anti* isomerism that has already been observed[6,7].

In order to confirm the above conclusion, the behaviour of peaks B and B′ was investigated in detail. Authentic DNPH–pyruvic acid gave the same peaks B and B′. Each component corresponding to peaks B and B′ was collected and re-subjected to HPLC. The two peaks B and B′ again appeared on the chromatogram, and the retention times were identical with those obtained before. However, a change in the ratio of the heights of peaks B and B′ was occasionally observed. The effect of time course on the changes which occurred in solution was then examined. When two aqueous ethanolic solutions of the DNPH derivative which gave different peak-height ratios immediately after preparation were allowed to stand for 2 weeks at room temperature, both solutions resulted in identical peak-height ratios. From the above facts, it was concluded that the compounds which gave peaks B and B′ were *syn* and *anti* isomers of DNPH–pyruvic acid. Similarly, α-ketobutyric acid or phenylpyruvic acid also gave two peaks and the ratio of the peak height of the second peak eluted to that of the first became greater with increasing bulk of the R group in the α-keto acid (R–COCOOH). It is suggested that the formation of the *syn* isomer becomes more favourable than that of the *anti* isomer due to the steric interaction between the R group and the dinitrophenyl group, and that the isomer with the shorter retention time is the *anti* form. This was confirmed by the nickel-complexation reaction in which, according to Katsuki and co-workers[7,8], the *syn* isomer of a DNPH–α-keto acid forms a coloured complex with a nickel ion but the *anti* isomer does not.

Fig. 2 shows the effect of water content on the isomerization of the *anti-*

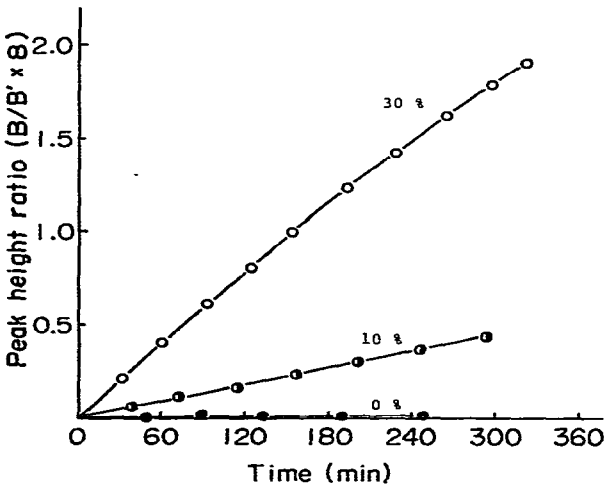



Fig. 2. Effect of water content on the isomerization of the *anti*-DNPH derivative of pyruvic acid at 40°.

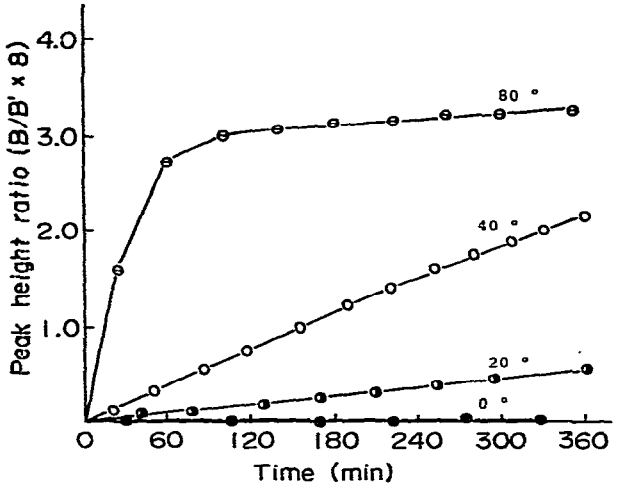



Fig. 3. Effect of temperature on the isomerization of the *anti*-DNPH derivative of pyruvic acid (ethanol content, 70%).

DNPH derivative of pyruvic acid which was obtained by recrystallization of a mixture of the isomers from aqueous methanol. The ratio of the peak heights of B and B' increased with increasing water content. These data indicate that water plays a significant role in the isomerization of the DNPH derivative. In order to obtain further evidence of this role, the following experiments were carried out. When an aqueous ethanolic solution of DNPH–pyruvic acid was warmed with α-ketoglutaric acid, DNPH–α-ketoglutaric acid was formed in the mixture. Similarly, the formation of DNPH–pyruvic acid was also observed on warming an aqueous ethanolic solution

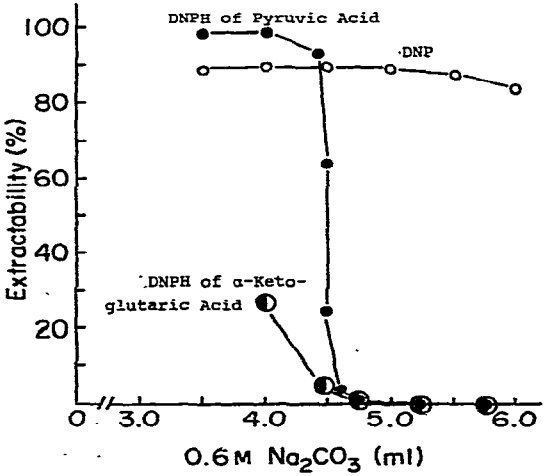



Fig. 4. The extractability of DNP, and of the DNPH derivatives of pyruvic acid and α-ketoglutaric acid, with chloroform compared with the volume of 0.6 *M* sodium carbonate added to the reaction mixture.

of DNPH–α-ketoglutaric acid with pyruvic acid. These data show that the DNPH derivatives are isomerized via hydrolysis as shown.

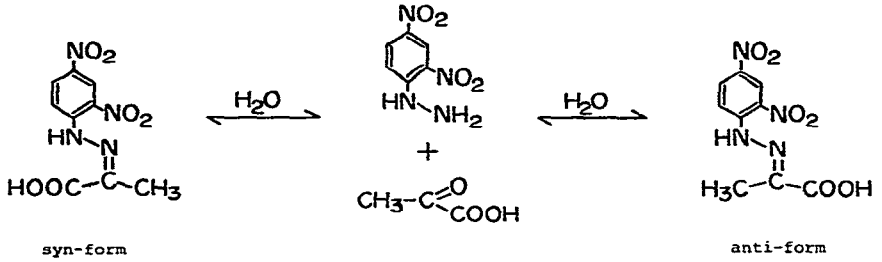
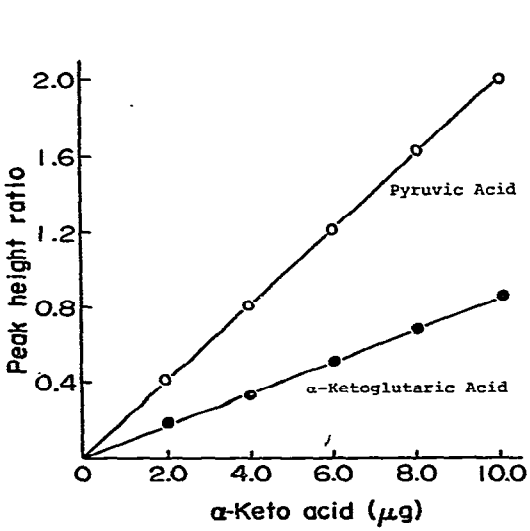



Fig. 3 shows that the isomerization is significantly depressed by cooling, despite the presence of water in the solution. Therefore, it is preferable to cool the mixture after the reaction in order for reliable determinations to be made.

Preliminary experiments showed that DNP reacted with α-keto acids in acidic media. Constant yields were obtained when the concentration of hydrochloric acid in the reaction mixture was greater than 0.5 *N*. There was no effect on the reaction when tungstate was used as a protein precipitant.

Fig. 4 shows the relations between the extractability of DNP, and of the DNPH derivatives of pyruvic and α-ketoglutaric acids, with chloroform and the quantity of 0.6 *M* sodium carbonate added to the reaction mixture. The DNPH derivatives could not be extracted with chloroform when more than 4.7 ml of 0.6 *M* sodium carbonate was added to the reaction mixture, but DNP was extractable even when 5.5 ml of 0.06 *M* sodium carbonate was added. These results were used to remove excess of DNP from the reaction mixture. It was also found that the DNPH derivatives could be easily extracted with ethyl acetate from acidic aqueous media.



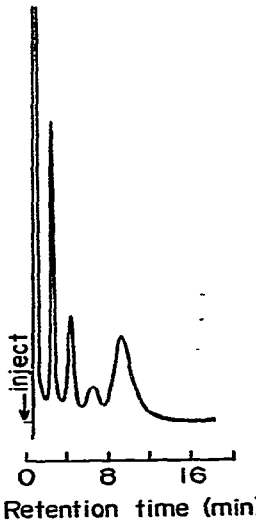




Fig. 5. Calibration graph for the determination of pyruvic acid and α-ketoglutaric acid.

Fig. 6. Liquid chromatogram obtained from normal serum sample. Operating conditions were as in Fig. 1.

TABLE I

RECOVERY OF PYRUVIC ACID (PA) AND α-KETOGLUTARIC ACID (α-KG) FROM SERUM

$\sigma_{PA} = 1.55\%$; $\sigma_{\alpha\text{-KG}} = 2.86\%$.

| *Amount added (μg)* | | *Amount found (μg)* | | *Recovery (%)* | |
|---|---|---|---|---|---|
| *PA* | *α-KG* | *PA* | *α-KG* | *PA* | *α-KG* |
| 4.00 | 2.00 | 2.96 | 1.51 | 74.0 | 75.7 |
| 4.00 | 2.00 | 2.85 | 1.54 | 71.2 | 77.1 |
| 4.00 | 2.00 | 2.96 | 1.66 | 74.1 | 83.0 |
| 4.00 | 2.00 | 2.90 | 1.52 | 72.4 | 76.0 |
| 4.00 | 2.00 | 3.02 | 1.51 | 75.6 | 75.5 |
| 4.00 | 2.00 | 3.04 | 1.52 | 75.9 | 76.1 |
| 4.00 | 2.00 | 2.96 | 1.50 | 74.1 | 75.2 |
| 4.00 | 2.00 | 2.98 | 1.61 | 74.4 | 80.7 |
| | | | | av. 74.0 | 77.4 |

Trimellitic acid, which is moderately soluble in water, was suitable as an internal standard. The effect of the concentration of sodium chloride in the aqueous phase on the extractability of the DNPH derivatives and trimellitic acid with ethyl acetate was investigated, and a constant extraction ratio of the two components was obtained by addition of 4 g of sodium chloride.

Fig. 5 illustrates the calibration graphs obtained by the overall procedure. When peak-height ratios of the DNPH derivatives of pyruvic acid and α-ketoglutaric acid relative to an internal standard were plotted against the amount of each α-keto acid, a good linear relation was obtained for at least the concentration range shown in Fig. 5.

Table I shows the percentage recoveries and the standard deviations obtained for eight replicate determinations made on an identical serum in which 4.00 μg of pyruvic acid and 2.00 μg of α-ketoglutaric acid were spiked. Fig. 6 shows a chromatogram obtained by the recommended procedure for the determination of pyruvic acid and α-ketoglutaric acid in serum. No interference from other peaks was observed.

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