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Note

Gas chromatographic separation of Krebs-cycle metabolites

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Gas–liquid chromatographic (GLC) techniques have been used for the evaluation of citric acid-cycle metabolites by several authors[1–15]. The simultaneous determination of Krebs-cycle acids by GLC is preferable to conventional enzymic methods because of the reduction in analysis time; these acids are usually analysed as methyl[1–8] or silyl[9–12] derivatives. Methylation has been performed with methanol and hydrogen chloride[1,2], methanol and boron trifluoride[3,4] or diazomethane[1–3,5–8], but, despite the different esterification conditions used, no single method had been reported in which all the compounds are methylated simultaneously. Simple esterification of acids containing double bonds or oxo groups is unsuitable because it gives rise to multiple peaks[4–6].

Silylation associated with oximation appears to be non-destructive[9–11]; nevertheless, only a few papers report the GLC analysis of Krebs-cycle acids as oximes and silyl derivatives[10], methoximes and silyl derivatives[11] or quinoxalone and silyl derivatives[12]. A few communications report the levels of some citric acid-cycle acids extracted from tissues and measured as methyl esters (not as silyl derivatives); recoveries were variable and low[13–15].

Thus, there is a need of a method for extracting citric acid-cycle acids and evaluating them as methoxime and silyl derivatives by GLC; this paper is a preliminary report of such a method.

MATERIALS

Standard acids (succinic, fumaric, malic, oxalacetic, citric, isocitric, *cis*-aconitic and 2-oxoglutaric) were obtained from BDH (Poole, Great Britain) and Biochemia (Milan, Italy). The solvents were purchased from Carlo Erba (Milan, Italy) and distilled before use. The silylating reagents [trimethylchlorosilane, hexamethyldisilazane and bis(trimethylsilyl)trifluoroacetamide] were obtained from Applied Science Lab. (State College, Pa., U.S.A.). For GLC, a Hewlett-Packard Model 5750 gas chromatograph, with a hydrogen flame detector, was used; the coiled column (6 ft. × 5 mm I.D.) was packed with Gas-Chrom P coated with 5% of SE-30. Purified air, nitrogen and hydrogen for GLC were obtained from SIAD (Bergamo, Italy).

METHODS

Preparation of derivatives[16]

Oximation. The standard oxo acid (0.02–0.1 mg) was dissolved in 0.2 ml of pyridine and allowed to react with 0.3 mg of methoxylammonium chloride for 1 h in a PTFE-lined screw-capped tube at room temperature. After removal of the reagents, the product was silylated.

Silylation. The standard carboxylic acid (0.02–0.1 mg) was dissolved in a mixture of pyridine (200 μl), trimethylchlorosilane (50 μl) and hexamethyldisilazane (100 μl), or bis(trimethylsilyl)trifluoroacetamide (100 μl) and pyridine (200 μl), and allowed to react for 1 h at room temperature. An internal standard (3,3-dimethylglutaric acid or tricarballylic acid) was previously added to the reaction mixture.

Gas chromatography

Because some of the derivatives were sensitive to water, the final reaction mixture was injected directly on to the column without removal of reagents (excess of reagent is necessary to ensure quantitative reaction of molecules having multiple functions).

The operating conditions of the gas chromatograph were generally: column temperature, 130° for dicarboxylic acids and 180° for tricarboxylic acids; detector temperature, 240°; inlet temperature, 260°; gas flow-rate, about 20 ml/min.

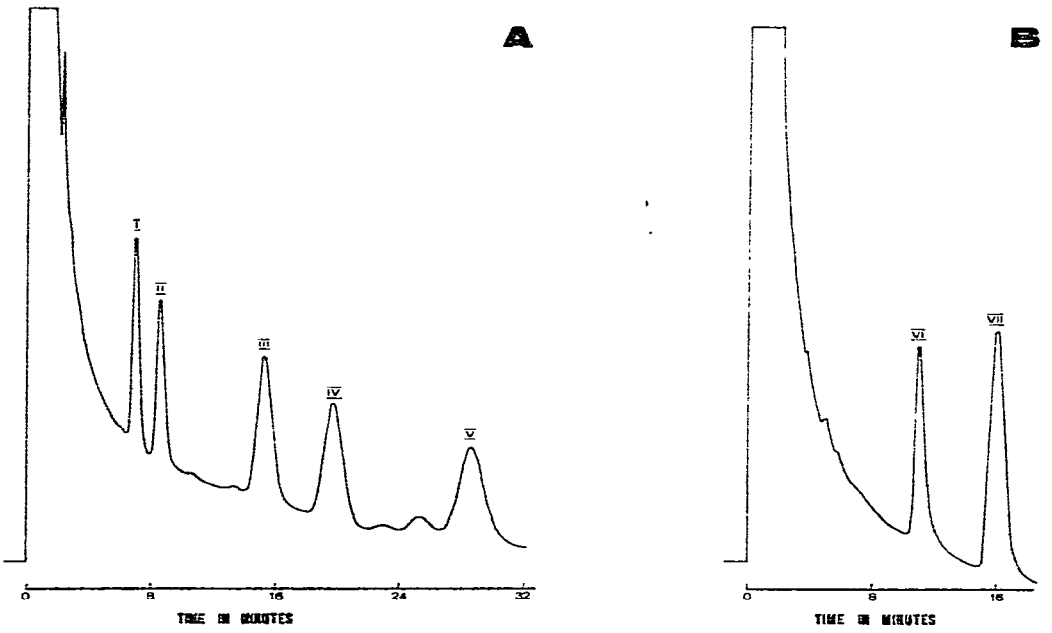

Fig. 1. Separation of microgram amounts of citric acid-cycle metabolites as silyl or methoxime–trimethylsilyl derivatives on an SE-30 column. The acids are located in the following order: succinic(I), fumaric(II), oxalacetic(III), malic(IV), 2-oxoglutaric(V), *cis*-aconitic(VI) and citric(VII). The column temperature was 130° for the dicarboxylic acids (A) and 180° for the tricarboxylic acids (B).

RESULTS AND DISCUSSION

The di- and tricarboxylic acids of the Krebs cycle are well separated by isothermal GLC at 130° and 180°, respectively (see Fig. 1); their relative retention times are shown in Tables I and II. The partial overlapping of peaks for citric and isocitric acids has been reported by other authors[11]. The relative retention times of the compounds studied were highly reproducible over a period of more than 12 months.

TABLE I

RETENTION TIMES OF DICARBOXYLIC ACIDS, AS SILYL OR METHOXIME–SILYL DERIVATIVES, RELATIVE TO 3,3-DIMETHYLGLUTARIC ACID ON SE-30 COLUMN

The retention time of dimethylglutaric acid was 13.76 min at 130°.

Compound	*Relative retention time*
Succinic acid	0.51
Fumaric acid	0.62
3,3-Dimethylglutaric acid	1.00
Oxalacetic acid (methoxime)	1.11
Malic acid	1.43
2-Oxoglutaric acid (methoxime)	2.08

TABLE II

RETENTION TIMES OF TRICARBOXYLIC ACIDS, AS SILYL DERIVATIVES, RELATIVE TO TRICARBALLYLIC ACID ON SE-30 COLUMN

The retention time of tricarballylic acid was 9.36 min at 180°.

Compound	*Relative retention time*
Tricarballylic acid	1.00
cis-Aconitic acid	1.07
Citric acid	1.57
Isocitric acid	1.45

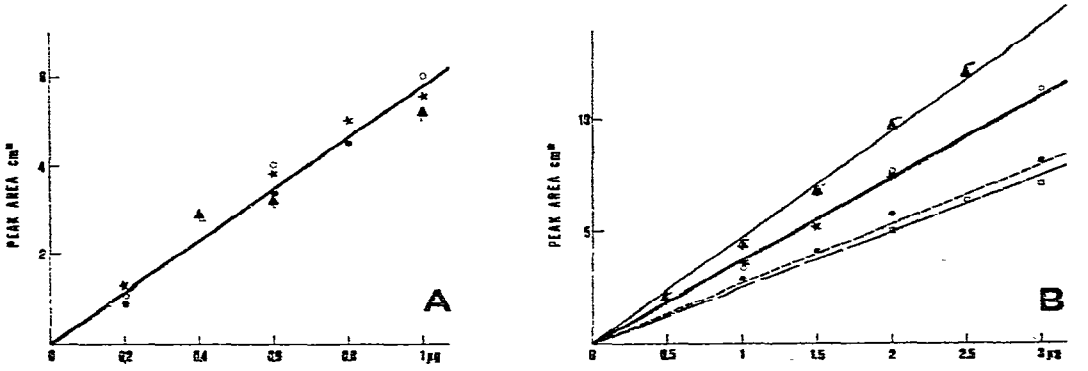

Fig. 2. Relationship between peak area and concentration of different amounts of (A) succinic (*), fumaric (●), malic (△) and 3,3-dimethylglutaric (○) acids; and (B) oxalacetic (●), 2-oxoglutaric (□), *cis*-aconitic (*), citric (▲) and isocitric (○) acids.

Under the experimental conditions described, conversion of the parent compounds into the derivatives was complete, and no extraneous peaks were observed.

Detector response to increasing quantities of samples was rectilinear (see Fig. 2), and submicrogram amounts could easily be detected. The use of 3,3-dimethylglutaric and tricarballylic acid as internal standards allows peak identification and permits recovery calculations when the chemicals are added to samples at the beginning of the extraction procedure.

For these reasons, the GLC procedure described here is suggested as suitable for the evaluation of Krebs-cycle acids extracted from biological materials.

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