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Note

Separation of tricarboxylic acid cycle acids and other related organic acids in insect haemolymph by high-performance liquid chromatography

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The tricarboxylic acid (TCA) cycle is the final common pathway of oxidative catabolism in aerobic cells, and its acid components can provide valuable information as to the metabolic status of an organism. Even so, analysis of such a complex mixture of keto and hydroxy acids has proved difficult. The use of paper chromatography[1,2], thin-layer chromatography[3] and isotachophoresis[4] have met with limited success. Gas chromatography (GC) has provided best separations with a high degree of sensitivity, and has been used widely in the analysis of biological samples[5–11]. However, derivatization of the acids to either their trimethylsilyl or oxime–trimethylsilyl derivatives renders the keto acids unstable[10,12], preventing the simultaneous analysis of TCA cycle acids under these conditions.

More recently, application of ion-moderated partition chromatography to organic acid analysis has allowed separation of all TCA cycle intermediates[13–15], and this has become the method of choice for determining organic acids in biological samples[16–18]. Even more appealing is the fact that the acids, which are mostly found in aqueous solutions in practice, do not require derivatization, but can be analysed directly, resulting in accurate quantification. However, separations achieved so far have not allowed full application of this technique to the study of TCA cycle and related acid metabolites as indicators of adaptive or stress metabolism in invertebrate systems[9,11,19–22]. Study of such systems demands simultaneous separation of not only TCA cycle intermediates, but also other acid metabolites (*e.g.* propionate, pyruvate, lactate, acetate and glyoxylate) produced either as a consequence of environmental stress, or by utilization of alternative carbon sources[11,19,20]. The present research was undertaken to establish the conditions required to achieve these separations on a commercially available high-performance liquid chromatography (HPLC) column, using insect haemolymph as a model invertebrate sample.

EXPERIMENTAL

Materials

Analytical grade sulfuric acid and acetic acid were purchased from Mallinck-

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rodt. Organic acid standards were purchased from Sigma and used without further purification. Distilled water was purified with a Sybron/Barnstead column purification system fitted with deionizing columns, an organics column, and a 0.2-μm filter. dissolved gases were removed from all solvents to be used in the mobile phase by placing them under vacuum.

Instrumentation

The HPLC system consisted of two altex 110A pumps with a 20-μl Rheodyne 7125 loop injection valve coupled to an Aminex HPX-87H organic acid column (Bio-Rad Labs.) fitted with a plexiglass water jacket (Alltech). Column effluent was passed through an Isco V4 UV detector at 210 nm. Elution conditions were controlled by an Axxiom 710 controller and data analyzed with a Shimadzu C-R1B integrator.

Methods

Haemolymph was collected from the 5th instar larvae of the silkworm *Bombyx mori*, deproteinised and the organic acid fraction collected and purified as previously described[10]. The column was conditioned with 0.01 *N* sulfuric acid at a pumping rate of 0.2 ml/min for at least 24 h prior to analysis, then allowed to equilibrate with the required mobile phase for 4 h before injection of standard or sample acids. Effects of changes in concentration of acid in the mobile phase (0.003–0.015 *N* sulfuric acid), pumping rate (0.5–0.6 ml/min) and column temperature on resolution of standard acids were tested. Standard acids were chromatographed individually to determine retention times and then in mixtures. Haemolymph acids were identified by co-chromatography with standards and by enzymatic procedures[10,19].

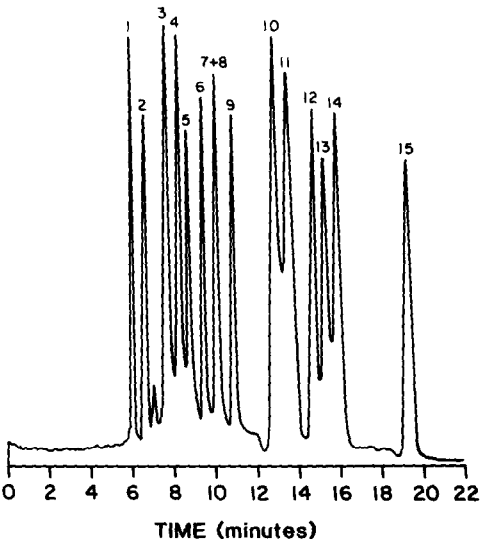

Fig. 1. Separation of organic acids on Aminex HPX-87H cation-exchange resin. 1 = Oxalic, 0.8 μg; 2 = oxaloacetic, 3 μg; 3 = α-ketoglutaric, 2 μg; 4 = citric, 3 μg; 5 = isocitric, 7 μg; 6 = pyruvic, 3 μg; 7 = glyoxylic, 7 μg; 8 = malic, 7 μg; 9 = *cis*-aconitic, 0.2 μg; 10 = succinic, 30 μg; 11 = lactic, 10 μg; 12 = formic, 10 μg; 13 = acetic, 10 μg; 14 = fumaric, 0.2 μg; 15 = propionic, 30 μg. Conditions: mobile phase, 0.004 *N* sulfuric acid; flow-rate 0.6 ml/min; column temperature, 41°C; UV detector, 210 nm; injection volume, 20 μg.

RESULTS AND DISCUSSION

Best resolution of TCA cycle intermediates was achieved using low acidity (0.004–0.006 *N* sulfuric acid) and high temperatures. For example, all TCA cycle intermediates were adequately resolved using 0.006 *N* sulfuric acid in the mobile phase, at a pumping rate of 0.6 ml/min and a column temperature of 41°C. However, under these conditions pyruvate eluted with malate and glyoxylate. With 0.004 *N* sulfuric acid in the mobile phase, we achieved the resolution shown in Fig. 1, which shows resolution of TCA cycle intermediates as well as oxalic, lactic, pyruvic, formic, acetic, and propionic acids. By decreasing the temperature and flow-rate separation of glyoxylate and malate was achieved, but these changes also resulted in decreased separation between lactate and succinate and acetate and fumarate (Fig. 2). further decreases in temperature resulted in fusion of these latter two pairs of peaks into two peaks, but also enhanced resolution of glyoxylate and malate. Conversely, increasing column temperature caused greater separation of succinate and lactate, but resulted in a loss of separation between earlier eluting acids.

All the acids we tested were stable under these conditions, with the exception of oxaloacetic acid, which gave two elution peaks, one of which was eluted as shown in Figs. 1 and 2, while the other co-chromatographed with pyruvate. The latter peak was apparently due to a keto–enol shift in aqueous solution. This isomerization would appear to be problematic, but since the ratio between the two peaks was constant at a given pH, it was possible to calculate the size of the oxaloacetate peak which co-chromatographs with pyruvate. The area of the pyruvate peak was then obtained by subtraction.

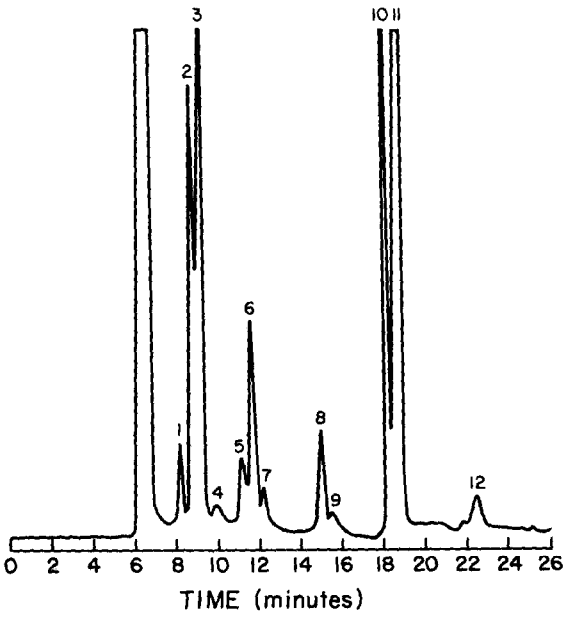

Fig. 2. Separation of organic acids on Aminex HPX-87H cation-exchange resin. The order of elution and amount of each acid as in Fig. 1. Conditions as in Fig. 1, except: column temperature, 33°C; flow-rate, 0.5 ml/min.

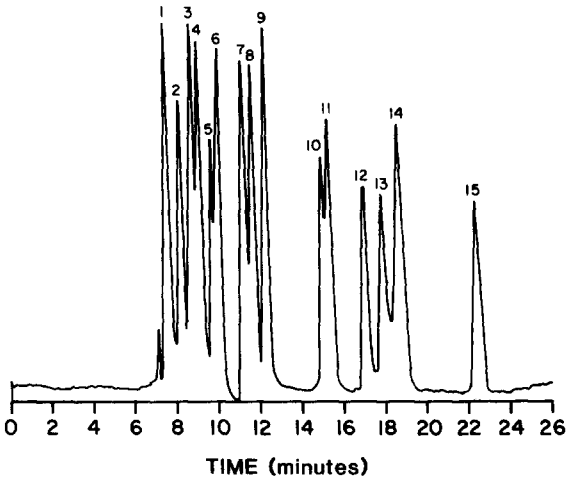

Fig. 3. Separation of organic acids in the haemolymph of the 5th instar larvae of *B. mori* on aminex HPX-87H cation-exchange resin. 1 = Oxaloacetic; 2 = α-ketoglutaric; 3 = citric; 4 = pyruvic; 5 = glyoxylic; 6 = malic; 7 = *cis*-aconitic; 8 = succinic; 9 = lactic; 10 = unknown; 11 = fumaric; 12 = propionic. Conditions as in Fig. 2.

A typical separation of organic acids occurring in the haemolymph of 5th instar *B. mori* larvae is presented in Fig. 3. We specifically chose to analyse this material due to its complex mixture of organic acids[23–25]. In agreement with published data[23,24] TCA cycle acids are the main organic acid constituents in *B. mori*, showing a similar acid pattern to that found in mosquito haemolymp[11]. We were also able to confirm the presence of *cis*-aconitate and of pyruvate, the latter of which had not been clearly established[25]. We were, however, unable to confirm the identity of peak 10, which is probably a mixture of acetic and acetoacetic acids[24]. Application of these procedures to other invertebrate systems, for the study of anaerobic metabolism[21] or adaptations to normal aerobic pathways[19,20] promises to be highly successful, since they allow the simultaneous analysis of organic acid components in a singel sample.

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