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Note

New method of purification of perchloric acid extracts for quantitative gas chromatography of some Krebs cycle acids

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It is evident from a great number of reviews [1–5] and detailed publications [6–8] that there is no generally accepted method for the quantitative extraction of organic acids from small amounts of biological tissues for derivatization and gas chromatography (GC). As for the quantitative extraction of a hydrophilic acid, such as citric acid, from whole blood, there is no detailed publication at all, to the best of our knowledge.

In order to obtain a total organic acid extract, ethanol deproteinization is the preferred method for most purposes [4]. This leads, however, to coprecipitation of citric acid. Deproteinization with ice-cold perchloric acid does not have this disadvantage. Unfortunately, there is no method in the literature for quantitative purification, adapted to this deproteinization method.

We believe that the extraction of the crystalline residue of the dried perchloric acid extracts with acidified, water-saturated diethyl ether constitutes such a method.

EXPERIMENTAL

Materials

Bis(trimethylsilyl)trifluoroacetamide (Merck, Darmstadt, F.R.G.), hydroxylamine hydrochloride, diethyl ether (Berlin-Chemie, Berlin-Adlershof, G.D.R.), pyridine (VEB Teerdestillation, Erkner, G.D.R.), hydrochloric acid, sodium chloride, sulphuric acid and methanol (VEB Laborchemie, Apolda, G.D.R.) of

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analytical-reagent grade were used. All solvents were redistilled. The diethyl ether was saturated with 6 *M* hydrochloric acid and bubbled with gaseous hydrogen chloride from sodium chloride and sulphuric acid. Standards of organic acids were purchased from Serva (Heidelberg, F.R.G.), Merck and Lachema (Brno, Czechoslovakia). [1,4-^{14}C]Succinic acid (0.29 GBq/mmol) and [1-^{14}C]malic acid (0.24 GBq/mmol) were obtained from Techsnabexport (U.S.S.R.) and [1,5-^{14}C]citric acid (4.11 GBq/mmol) and 2-keto[5-^{14}C]glutaric acid (0.33 GBq/mmol) from the Radiochemical Centre (Amersham, U.K.).

Deproteinization

For deproteinization, 6 ml of rabbit blood cell suspensions in an Eagle solution were added to an equal volume of chilled 8% perchloric acid, containing 15 μg/ml tartaric acid (internal standard). After washing of the pellet with 4 ml of 4% perchloric acid, filtration, neutralization with 5 *M* potassium carbonate and standing for 30 min at −20°C, the combined supernatants were evaporated in a rotary evaporator at 26°C.

Extraction and derivatization

A 3-ml volume of acidified, water-saturated diethyl ether was added to the crystalline residue. The conical flask was shaken vigorously. Gaseous hydrogen chloride was bubbled through until an acid pH was reached. After 10 min the diethyl ether was removed. The extraction procedure was repeated five times without further bubbling of hydrogen chloride, because the pH remained acid. The combined diethyl ether fractions were evaporated under a stream of nitrogen at 4°C. The remaining residue was dried under reduced pressure (rotary evaporator) for 5 min at 26°C and resuspended in 1 ml of methanol. Aliquots of 0.2–0.6 ml were placed into Wheaton vials, evaporated under nitrogen at 4°C and taken up in 0.1 ml of pyridine containing hydroxylamine hydrochloride (2 g per 100 ml). Then 0.2 ml of bis(trimethylsilyl)trifluoroacetamide was added. Before injection of an aliquot of 15–30 μl, the vials were incubated for 1 h at 90°C.

Gas chromatogaphy

A modified GCHF 18-3 gas chromatograph (Chromatron Berlin) with a flame ionization detector was used. The guard column and glass column (1 m×2 mm I.D.) were packed with 3% SE-30 on 80–100 mesh Chromosorb W HP (Johns-Manville). The injection port and detector temperatures were ca. 270°C, the nitrogen flow-rate was 50 ml/min, and the hydrogen and air flow-rates were maintained at 20 and 600 ml/min, respectively. Temperature programming was from 100 to 250°C at 32°C/min.

Radioactivity measurements

Radioactivity was measured in duplicate in dioxane scintillator with a liquid scintillation counter using a Packard 3380 A (Packard Instruments, U.S.A.).

RESULTS AND DISCUSSION

In order to verify the purification procedure, we analysed erythrocyte suspensions substituted with 0.2–1.0 μmol of standard acids. These amounts represent 1 g of tissue (wet weight). The recovery of the acids after deproteinization and purification was studied via addition of ^{14}C-labelled acids. The amounts recovered of succinate, malate, 2-oxoglutarate and citrate were 87 ± 2, 88 ± 2, 84 ± 2 and $83 \pm 2\%$, respectively. The fact that citric acid gives the same results as the other acids is a clear advantage in comparison with all the other extraction methods, employing two liquid phases [9]. When the diethyl ether was not water-saturated, or not acidified sufficiently, the recoveries were substantially lower. Losses of acids also occurred if the samples were evaporated at elevated temperatures [6].

Diethyl ether should dissolve organic acids selectively, thus determining the quality of the purification step [10]. The comparison of the separation profiles of extracts from washed erythrocytes with these of the substituted ones shows that none of the trimethylsilyl (TMS) ester peaks of the Krebs cycle acids interferes with peaks of other origin. Only phosphoric acid, which is coextracted to some extent, makes it difficult to quantify succinic acid (Fig. 1). The small peak eluting at the same time as TMS-malate is derived entirely from malate remaining in the erythrocytes after the washing procedure. This was confirmed enzymatically.

There are no difficulties in the silylation of the purified extracts, unlike the anion-exchange procedure for purifying perchloric acid extracts [4]. All relevant acids were silylated to the same extent.

Quantification was carried out with the help of typical response curves, using tartaric acid as internal standard. The accuracy and precision of the entire procedure, including GC analysis, was characterized by the agreement between the

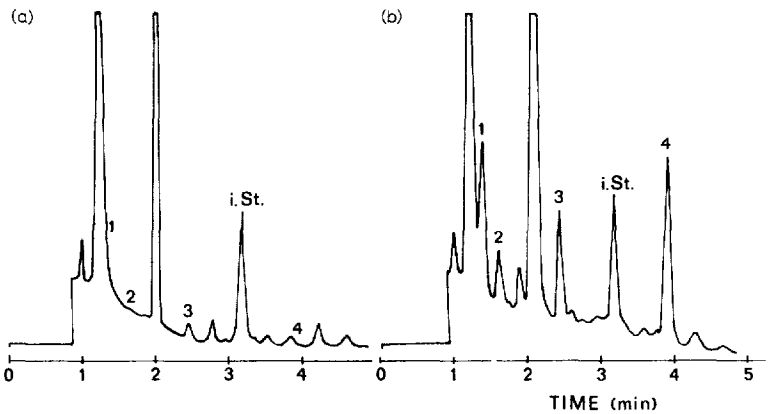

Fig. 1. GC separation of TMS derivatives of organic acids after extraction from washed erythrocytes (a) and washed erythrocytes substituted with standard acids (b). The following compounds were identified: succinate (1), fumarate (2), malate (3), tartrate (internal standard, i.St.) and citrate (4). The amount of each acid injected was ca. 3 nmol.

TABLE I

CONCENTRATIONS OF KREBS CYCLE ACIDS IN RETICULOCYTE-RICH ERYTHROCYTE SUSPENSIONS FROM RABBIT BLOOD

The cells were incubated for 15 min in a glucose-containing (5 m*M*) electrolyte medium, pH 7.4. The concentrations are expressed as nmol per ml reticulocytes; $n = 3$.

Compound	Concentration (mean ± S.D.)
Succinate	105 ± 26
Fumarate	72 ± 16
Malate	275 ± 62
Citrate	131 ± 3

measured amount of acids and the expected value. We obtained values of ca. 101 ± 7% for all the acids mentioned.

Another advantage of solid-phase extraction is that it is less time-consuming than the chromatographic purification methods. An application of the method is shown in Table I. In addition to blood, this method should be applicable to all other biological materials and tissues.

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