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Note

Ion-exchange extractive alkylation of organic acids

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Gas chromatography—mass spectrometry (GC—MS) is the most widely used method for the profiling of urinary organic acids. The isolation of the acids prior to GC—MS is usually carried out either by anion-exchange chromatography (most often with DEAE-Sephadex extraction [1]) or by solvent extraction [2]. Organic acids are then volatilized by derivatization. Because of their ease of preparation, trimethylsilyl (TMS) derivatives are usually used, although methyl derivatives have several advantages for GC and GC—MS, viz., better volatility, lower molecular weight, stability in the presence of water and more easily interpretable mass spectra.

Recently, extractive alkylation has been used for the extraction of oxo acids and their derivatization prior to GC—MS [3], based on extraction of the acids as an ion pair formed with tetramethylammonium hydroxide (TMAH) and subsequent methylation with methyl iodide, where the TMAH acts as a catalyst. We describe here a similar method based on the extraction of organic acids by an anion exchanger with TMAH groups followed by in situ methylation with methyl iodide. The evaporation step between the extraction and methylation is avoided so that the procedure permits rapid and quantitative sample preparation for GC and GC—MS analysis.

EXPERIMENTAL

Chemicals

Ethoxylammonium chloride (Eastman Kodak, Rochester, NY, U.S.A.), N-methyl-N-nitroso-*p*-toluenesulphonamide and methyl iodide (Merck, Darmstadt, F.R.G.), hydrochloric acid, sodium hydroxide and potassium hydroxide of analytical-reagent grade (Lachema, Brno, Czechoslovakia) were used. Ostion

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AT 0807, particle size 0.16—0.32 mm (Spolchemie, Ústi nad Labem, Czechoslovakia) as a strongly basic anion exchanger was used in the OH^- form (TMAH groups on styrene—divinylbenzene resin, similar to Dowex 1). Before use, the anion exchanger was purified by repeated washing with 1 *M* sodium hydroxide solution, 1 *M* hydrochloric acid, hot redistilled water, ethanol and acetone. Standards of organic acids were purchased from Merck, Sigma (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland). All solvents were of chromatography grade (Merck).

Preparation of the extraction columns

A suspension of the anion exchanger in the water was pipetted into a 5-ml disposable syringe with a piece of the filter paper inserted at the bottom to form a 1-ml (1.2 × 0.9 cm) column bed and then washed with 2 ml of water.

Extraction and derivatization by ion-exchange extractive methylation

A 1-ml volume of either urine or a mixture of organic acid standards (2 mg each in 50 ml of water) was placed in a 5-ml glass tube and 20 μl of internal standard solution (malonic acid, 10 mg in 5 ml of water) were added. The sample was neutralized with a few drops of 5 *M* sodium hydroxide solution and 10 mg of ethoxylammonium chloride were added to form ethoxime derivatives of the oxo acids. After standing for 30 min at room temperature the sample was placed on the top of the extraction column, the column was washed twice with 1 ml of water and twice with 1 ml of methanol and the anion exchanger was removed and placed in the 5-ml stoppered glass tube. A 2-ml volume of acetone—methyl iodide (1:1) was added and the sample was left to stand for 60 min at 40°C. After the reaction, the anion exchanger was filtered off and the sample was concentrated to about 200 μl under a stream of nitrogen. A 5-μl volume of methyltridecanoate solution (20 mg in 1 ml of dichloromethane) was added as an internal standard and 1 μl of the sample was injected using the split injection technique.

GC—MS analysis

A 1020b GC—MS system (Finnigan-MAT, Sunnyvale, CA, U.S.A.) was used under the following conditions: gas chromatograph, Sigma 3b (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a 40 m × 0.32 mm I.D. BP-5 FSOT column (bonded SE-54 phase) (SGE, Victoria, Australia); injector in the split mode (splitting ratio 1:50) operated at 230°C; initial oven temperature, 60°C, final temperature 250°C, ramp-rate 10°C/min, initial time 2 min, final time 5 min; GC—MS interface, direct inlet at 260°C; manifold temperature, 70°C; electron energy, 70 eV; carrier gas, helium; flow-rate, 25 cm/s.

RESULTS AND DISCUSSION

The ion-exchange extractive alkylation is in principle a modification of the extractive alkylation used by various workers [4]. In contrast to ion-pair formation with TMAH, extraction with an anion exchanger containing TMAH groups offers several distinct advantages for the isolation of organic acids as a group, and it has been used for the extraction of organic acids from biological materials prior to GC and GC—MS [5, 6].

In the alkylation step the TMAH acts as a catalyst, after which it should be removed from the solution prior to GC [4]. Also in this step the TMAH group bonded on the resin offers the advantage of easy separation. We focused our attention on ion-exchange methylation using methyl iodide as this procedure proved to be very efficient for the extraction and methylation of several types of organic acids (Table I). For the organic acids studied, the recoveries of the whole procedure were 75—110%.

TABLE I

EFFICIENCY OF EXTRACTION AND METHYLATION OF ORGANIC ACIDS BY ION-EXCHANGE EXTRACTIVE METHYLATION

Five replicates.

Acid	Recovery (%)	Coefficient of variation (%)
Glycolic acid	75	14
Lactic adid	87	9.1
3-Hydroxybutyric acid	84	8.3
Methylmalonic acid	110	5.6
Succinic acid	99	2.7
Pyruvic acid	91	3.9
4-Hydroxyphenylacetic acid	94	7.1
Pimelic acid	102	6.7

Fig. 1 shows a reconstructed ion chromatogram (RIC) of a mixture of organic acids. Component A is the product of the reaction of two molecules of acetone under the conditions used. In subsequent studies we intend to find another solvent for efficient alkylation in place of acetone.

Fig. 2 shows the RIC of the urine from a patient with isovaleric acidaemia.

From this study, several conclusions can be drawn.

(i) The methylation of the carboxylic groups is fast and quantitative under the conditions used.

(ii) Hydroxy groups are methylated only if they are located on the aromatic ring or on the same carbon atom to which the aromatic ring is bonded (hydroxyphenyl acids, mandelic acid).

(iii) Amino groups in glycine conjugates are not methylated.

(iv) Organic acids with a double bond in the aliphatic chain are methylated on the carboxylic group only, in contrast to ring formation with diazomethane (fumaric acid).

We found that the anion exchanger should be specially purified before use. We used repeated washing with acetone, dichloromethane and methanol before a final wash with 1 *M* sodium hydroxide solution. In Fig. 1, however, several minor peaks are visible, probably due to impurities in the anion exchanger. A slightly less basic anion exchanger (QAE-Sephadex, trimethylaminohydroxypropylcellulose) proved not to be effective for the methylation of organic acids.

By omitting the evaporation step between the extraction and derivatization, the ion-exchange methylation of organic acids is a simple and fast procedure

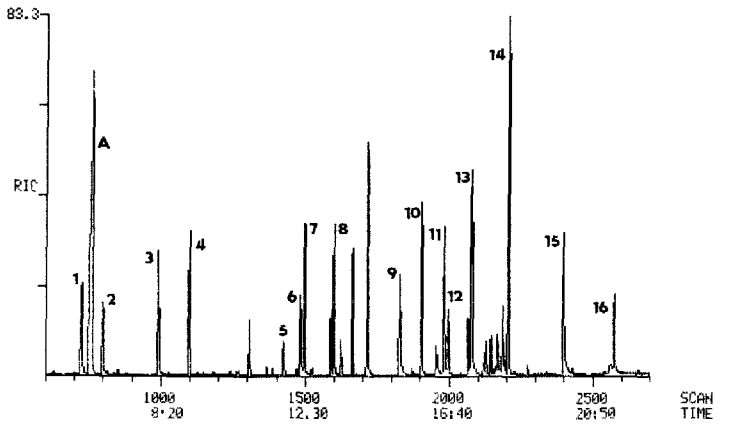

Fig. 1. Reconstructed ion chromatogram of a standard mixture of organic acids extracted and methylated using ion-exchange extractive methylation. Separation was carried out on a BP-5 (bonded SE-54) FSOT column with temperature programming from 60 to 250°C at 10°C/min. Organic acids were separated as their methyl (M) esters and/or ethers. Peaks: A = 4-hydroxy-4-methyl-2-pentanone; 1 = 2-hydroxybutyrate, 1-M; 2 = 3-hydroxybutyrate, 1-M; 3 = malonate, 2-M (internal standard); 4 = methylmalonate, 2-M, 5 = benzoate, 1-M; 6 = malate, 2-M; 7 = glutarate, 2-M; 8 = phenylacetate, 1-M; 9 = mandelate, 2-M; 10 = pimelate, 2-M; 11 = phenyllactate, 1-M; 12 = 2-hydroxyphenylacetate, 2-M; 13 = 4-hydroxyphenylacetate, 2-M; 14 = tridecanoate, 1-M (external standard); 15 = sebacate, 2-M; 16 = diphenylacetate, 1-M.

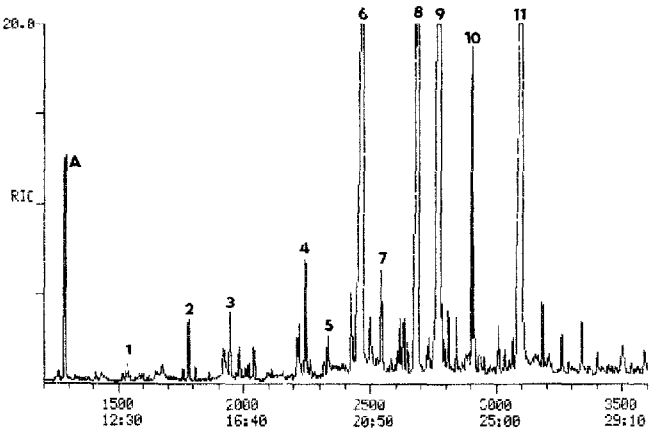

Fig. 2. Reconstructed ion chromatogram of organic acids extracted and methylated using ion-exchange extractive methylation from the urine of a patient with isovaleric acidaemia. Separation was carried out on a BP-5 (bonded SE-54) FSOT column with temperature programming from 60 to 250° C at 10° C/min. Organic acids were separated as their methyl (M) esters and/or ethers. Peaks: A = 4-hydroxy-4-methyl-2-pentanone; 1 = phosphate, 3-M; 2 = succinate, 2-M; 3 = benzoate, 1-M; 4 = glutarate, 2-M; 5 = 3-methylglutarate, 2-M; 6 = isovalerylglycine, 1-M; 7 = 2,5-furandicarboxylate, 2-M; 8 = citrate, 3-M; 9 = tridecanoate, 1-M (external standard); 10 = phthalate, diethyl ester; 11 = hippurate, 1-M.

that offers several advantages over the methods commonly used for the isolation of organic acids from biological materials. It also permits the use of the advantageous methyl derivatives in GC and GC—MS. A subsequent paper will describe in detail the experimental procedure, its various possibilities and its efficiency for some of the labile and difficult-to-extract acids.

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