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IMPROVED PROCEDURE FOR THE ANION-EXCHANGE ISOLATION OF URINARY ORGANIC ACIDS

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SUMMARY

DEAE-Sephadex equilibrated in 0.5 *M* triethylammonium acetate is suitable for the quantitative isolation of lactonisable organic acids. Mono-, di- and tricarboxylic acids can be eluted sequentially from DEAE-Sephadex by the use of 0.5 *M* triethylamine, 0.5 *M* triethylamine—0.1 *M* acetic acid, and 1.5 *M* pyridinium acetate.

INTRODUCTION

During the last few years, metabolic profiling has been particularly useful in the screening for organic acids in the urine of patients suffering from suspected inborn errors of metabolism and up till 1977, 23 new diseases have been discovered by this route [1]. The establishment of a successful metabolic profiling technique involves the following considerations: efficient and selective extraction of the compounds; rapid and complete derivatization of the compounds; separation and identification of the components of the mixture and interpretation of the data in terms of which compounds are not normally present or are present in abnormal amounts. Techniques of extracting organic acids from urine have been the subject of several studies [2—5]. Two principal methods for the isolation of the acidic constituents of urine, prior to derivatization and gas chromatography (GC), are currently in use [1]. The first is based on solvent extraction, usually with diethyl ether and/or ethyl acetate, the second is based on anion-exchange chromatography. The problem of choosing between the solvent-extraction and anion-exchange methods often arises. It has been the experience of several groups, that if the problem concerns screening for gross metabolic disorders, the detection of which does not require quantitative

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separation, then solvent extraction is applicable whilst for quantitative work the more time consuming anion-exchange procedures are essential.

The published ion-exchange procedure [5] involves removal of sulphate and phosphate as barium salts, ion exchange on DEAE-Sephadex, elution of the acids with pyridinium acetate buffer, lyophilization, silylation and GC. We recently applied the above procedure to the quantitative extraction and assay of some abnormal deoxyribose metabolites present in the urine of a child suffering from a new inborn error of metabolism, and found that the main metabolite, a lactonisable organic acid, could not be quantitatively recovered [6]. We now describe a modified anion-exchange procedure which is suitable for the quantitative isolation of lactonisable organic acids.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: 2-hydroxy-3-methylbutyric acid, Pfaltz and Bauer (Stamford, CN, U.S.A.); 2-hydroxyisovaleric acid and 2-hydroxy-3-methylvaleric acid, from Sigma (St. Louis, MO, U.S.A.); phenylacetic acid, adipic acid, pimelic acid, glutaric acid and citric acid, Merck (Darmstadt, G.F.R.); DEAE-Sephadex A-25, Pharmacia (Uppsala, Sweden); 3% SE-30 on Gas-Chrom Q, Applied Science Labs. (State College, PA, U.S.A.); bis(trimethylsilyl)trifluoroacetamide (BSTFA), Regis Chemical (Morton Grove, IL, U.S.A.).

Reagents

The 0.5 *M* and 1.5 *M* pyridinium acetate buffers were prepared as described by Thompson and Markey [5]. The 0.5 *M* triethylammonium acetate buffer was prepared by adding 69 ml of triethylamine to 29 ml of glacial acetic acid and diluting the resulting solution to 1 l with distilled water. The pH of the buffer was 5.8. The 0.5 *M* triethylamine buffer was prepared by diluting 69 ml of triethylamine to 1 l with distilled water. The pH of this solution was 12.1.

The 0.5 *M* triethylamine—0.1 *M* acetic acid buffer was prepared by mixing 69 ml of triethylamine and 6 ml of glacial acetic acid and diluting the resulting solution to 1 l with distilled water. The pH of this buffer was 11.5.

Solvent extraction of urinary organic acids

Sodium chloride (0.5 g) was added to 1 ml of urine, followed by sufficient hydrochloric acid to bring the pH to 1. The solution was then extracted twice with ethyl acetate (2 × 2 ml) and once with diethyl ether (2 ml) and the pooled organic phases evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried urinary extract was then silylated with 125 μl of BSTFA (60°C, 30 min) and an aliquot corresponding to 40 nmol creatinine injected onto the GC column. The derivatives were analysed on a 183 × 0.3 cm I.D. glass column of 3% SE-30 on 80—100 mesh Gas-Chrom Q. A helium flow-

rate of 25 ml/min was used and during the analysis the temperature was programmed from 80—220°C at 6°C/min.

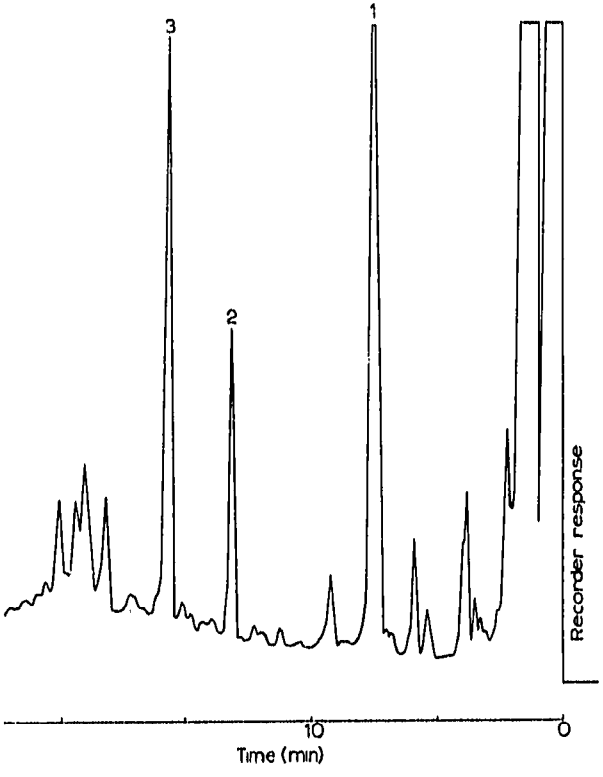
Isolation of urinary organic acids by ion-exchange chromatography

To an aliquot of urine (equivalent to 5 μmol of creatinine) in a centrifuge tube were added 2 ml of 0.1 *M* barium hydroxide. The contents were mixed, then centrifuged for 30 sec. The precipitate was washed with a further 2 ml of barium hydroxide and the supernatants combined. The supernatant was adjusted to pH 10 with 6 *N* hydrochloric acid and loaded on to a column containing 8 ml of DEAE-Sephadex A-25 which had been equilibrated in 0.5 *M* triethylammonium acetate. The column was washed with 40 ml of distilled water and the monocarboxylic acids were eluted with 40 ml of 0.5 *M* triethylamine. Glutaric acid (50 μl, 2000 μg) was added to the triethylamine effluent and the solution freeze-dried. The dried residue was dissolved in 1 ml of water and aliquots of this were redried and derivatized with BSTFA for GC analysis as described above.

Recovery of organic acids from DEAE-Sephadex

Standard solutions of concentration 5 mg/ml of each of the following acids in water or aqueous methanol were prepared: 2-hydroxy-3-methylbutyric, 2-hydroxyisovaleric, 2-methyl-3-methylvaleric, phenylacetic, adipic, pimelic and citric acids. Standard curves for each acid against glutaric acid as the internal standard were constructed by adding 50-μl, 100-μl and 200-μl aliquots of each acid solution, corresponding to 250, 500 and 1000 μg of acid, to separate screw-cap vials containing glutaric acid (100 μl, 500 μg). These solutions were freeze-dried and the dry residue was derivatized with BSTFA (200 μl, 60°C, 30 min) for GC analysis as described above. The ratios of peak heights of organic acids to glutaric acid were then plotted versus μg of each acid. All the calibration curves were linear over the concentration range used.

Aliquots (200 μl) of each of the above acid solutions, corresponding to 1000 μg of each acid, were combined and the resulting solution was adjusted to pH 10—11 with triethylamine. This resulting solution was applied to a column of DEAE-Sephadex (8 ml) equilibrated in 0.5 *M* triethylammonium acetate and the column was washed in turn with water (40 ml), 0.5 *M* triethylamine (40 ml), 0.5 *M* triethylamine—0.1 *M* acetic acid (40 ml) and 1.5 *M* pyridinium acetate. Glutaric acid (100 μl, 500 μg) was added to each fraction, after which the solutions were freeze-dried and derivatized with BSTFA (500 μl, 60°C, 30 min). Similarly, a second sample of the same solution was freeze-dried and derivatized without prior ion-exchange chromatography. The recovery of each organic acid in the triethylamine and triethylamine—acetate fractions were determined from the GC peak height ratios followed by interpolation from the respective calibration curves. The recoveries of the acids obtained in the two fractions have been summarized in Table I. The water wash did not contain any organic acids, whilst the pyridinium acetate eluate contained only the citric acid, confirming that all the other acids had been eluted with the other buffers.



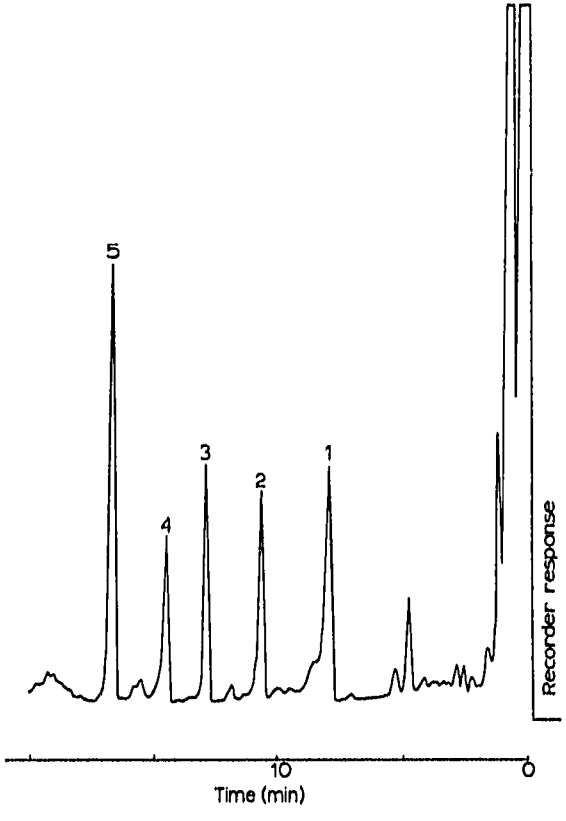


Fig. 1. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by solvent extraction. The numbered peaks were: (1) urea; (2) 2-deoxyerythropentono-1,4-lactone; (3) *p*-hydroxyphenylacetic acid.

Fig. 2. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by DEAE-Sephadex chromatography in 0.5 *M* pyridinium acetate buffer. The numbered peaks were: (1) phosphoric acid; (2) glutaric acid (internal standard); (3) 2-deoxyerythropentono-1,4-lactone; (4) 2-deoxyerythropentono-1,5-lactone; (5) 2-deoxyerythropentonic acid.

TABLE I

PERCENTAGE RECOVERIES OF ORGANIC ACIDS USING THE MODIFIED ANION-EXCHANGE METHOD ON DEAE-SEPHADEX

Compound	Recovery (%)
2-Hydroxy-3-methylbutyric acid	94
2-Hydroxyisovaleric acid	95
2-Hydroxy-3-methylvaleric acid	98
Phenylacetic acid	85
Adipic acid	97
Pimelic acid	91

Quantitation of 2-deoxyerythropentonic acid in urine

An aliquot of a standard solution (100 μl, 1000 μg) of 2-deoxyerythropentonic acid was added to normal urine corresponding to 10 μmol creatinine. Barium hydroxide (0.1 *M*, 2 ml) was added, centrifuged and the supernatants were removed. The urine solution was adjusted to pH 9—10 and applied to a column of DEAE-Sephadex (8 ml) equilibrated in 0.5 *M* triethylammonium acetate. The column was washed with 40 ml water and the acids were eluted with 40 ml of 0.5 *M* triethylamine. Glutaric acid (400 μl, 2000 μg) was added to the effluent, the solution was freeze-dried and derivatised with BSTFA (200 μl, 60°C, 30 min). The recovery of 2-deoxyerythropentonic acid was calculated from the GC peak height ratio versus the internal standard and interpolation of a standard curve. The recovery was 92—95%.

RESULTS AND DISCUSSION

The new method of isolation of organic acids was applied to a urine sample from a patient suffering from a defect in deoxyribose metabolism. The patient (J.S.) was an 18 months old boy, the only child of unrelated Greek parents. He was admitted to hospital because of poor feeding, vomiting and mild developmental delay. When the silylated ethyl acetate—diethyl ether extract of the patient's urine was analyzed by GC, large amounts of 2-deoxyerythropentono-1,4-lactone were observed (Fig. 1). It seemed likely that a considerable proportion of the lactone in the extract had arisen by lactonisation of 2-deoxyerythropentonic acid under the conditions used for solvent extraction. The rate and extent of lactonisation were not known however and the low extraction efficiencies of such hydrophilic compounds made quantitative studies difficult. In order to overcome this problem, the published ion-exchange procedure [5] was used for the quantitation of the new metabolite.

The GC profile of the silylated urinary extracts obtained by DEAE-Sephadex chromatography in 0.5 *M* pyridinium acetate buffer contained 2-deoxyerythropentonic acid, 2-deoxyerythropentono-1,4-lactone and 2-deoxyerythropentono-1,5-lactone (Fig. 2). The relative ratios of the three compounds obtained in various fractions were not constant, but varied considerably. The reason for the large variation in the extent of lactonisation following elution from DEAE-Sephadex is not known. This problem has already been encountered by other workers when measuring urinary tetronic and deoxy-

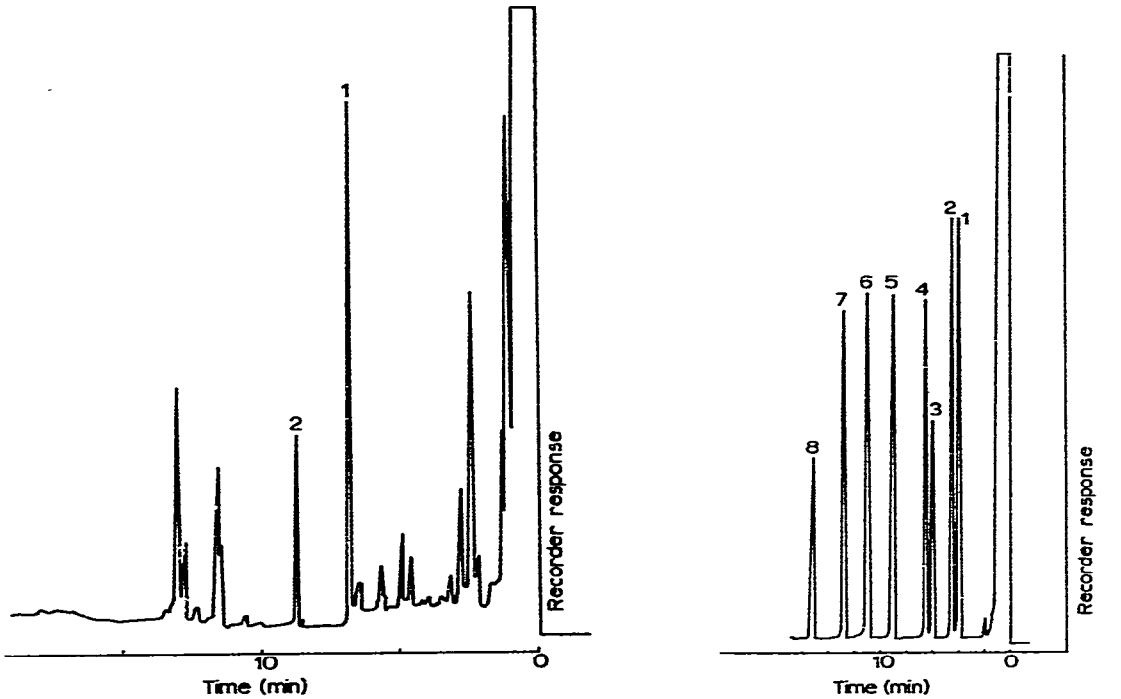
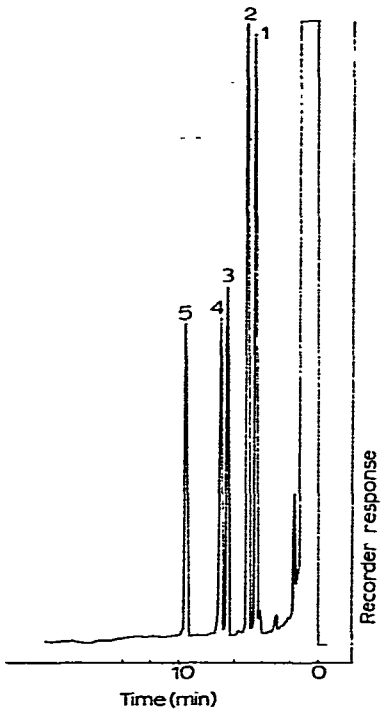


Fig. 3. GC trace of the silylated urinary extract from patient J.S. The organic acids were isolated from urine by the modified anion-exchange chromatography on DEAE-Sephadex. The numbered peaks were: (1) glutaric acid (internal standard); (2) 2-deoxyerythropentonic acid.

Fig. 4. GC trace of the silylated organic acid mixture used prior to DEAE-Sephadex chromatography. Peaks: (1) 2-hydroxy-3-methylbutyric acid; (2) 2-hydroxyisovaleric acid; (3) 2-hydroxy-3-methylvaleric acid; (4) phenylacetic acid; (5) glutaric acid (standard); (6) adipic acid; (7) pimelic acid; (8) citric acid.

tetronic acids [7]. We have now eliminated this problem by chromatographing the organic acids on DEAE-Sephadex equilibrated with 0.5 *M* triethylammonium acetate and eluting the 2-deoxyerythropentonic acid with a higher pH buffer. In this way the acid could be recovered in 92—95% yield, without lactonisation (Fig. 3). The modified procedure was used to analyse a standard mixture of carboxylic acids normally found in urine (Fig. 4) and it was shown that the mono-, di- and tricarboxylic acids could be eluted sequentially from the anion-exchange resin by the sequential use of three buffers, 0.5 *M* triethylamine (Fig. 5), 0.5 *M* triethylamine—0.1 *M* acetic acid (Fig. 6) and 1.5 *M* pyridinium acetate.

As well as providing improved recovery of sugar acids and other compounds which can undergo lactonisation, the use of this ion-exchange method has a number of additional advantages over the previously published procedure. For example in cases where patients are found to excrete novel compounds in the urine, the sequential elution procedure on DEAE-Sephadex when combined with GC, can be used to quickly determine the number of carboxyl groups in an unknown organic acid. The sequential elution process can then be repeated



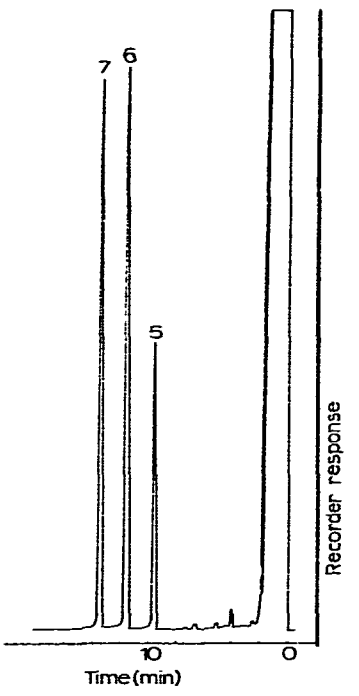


Fig. 5. GC trace of the silylated organic acids eluted from DEAE-Sephadex with 0.5 *M* triethylamine. Peaks: (1) 2-hydroxy-3-methylbutyric acid; (2) 2-hydroxyisovaleric acid; (3) 2-hydroxy-3-methylvaleric acid; (4) phenylacetic acid; (5) glutaric acid (standard).

Fig. 6. GC trace of the silylated organic acids eluted from DEAE-Sephadex with 0.5 *M* triethylamine—0.1 *M* acetic acid subsequent to elution with 0.5 *M* triethylamine. Peaks: (5) glutaric acid (standard); (6) adipic acid; (7) pimelic acid.

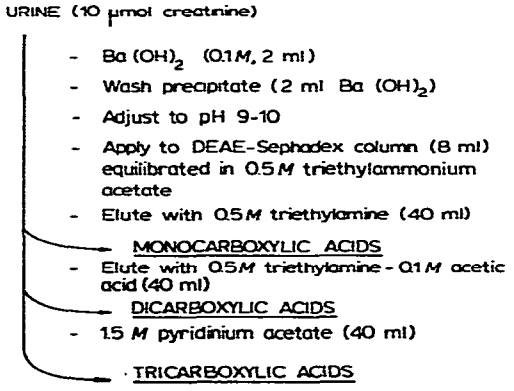


Fig. 7. Flow chart outlining modified anion-exchange chromatography on DEAE-Sephadex.

on a large scale as a step in the purification of such organic acids for subsequent structure determination. The modified ion-exchange procedure (Fig. 7) is particularly valuable for the quantitation of metabolites where the simplification of the urinary profile is of significant benefit.

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