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Note

Rapid and simplified extraction procedure for gas chromatographic—mass spectrometric profiling of urinary organic acids

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The application of gas chromatography (GC) and combined gas chromatography—mass spectroscopy (GC—MS) for the profiling of urinary organic acid metabolites has led to the detection of several new inborn errors of metabolism [1]. The isolation of the organic acids from urine prior to GC is usually done either by anion-exchange chromatography or by solvent extraction techniques. Because ion-exchange methods are more laborious and time consuming, solvent extraction is normally preferred for metabolite screening. We now describe a more rapid and simplified solid-phase extraction procedure for urinary organic acids.

EXPERIMENTAL

Materials and methods

Solid phase extraction tubes (Jetubes), of 3-ml capacity, were purchased from Manhattan Instr. (Santa Monica, Calif., U.S.A.). J.T. Baker (Phillipsburgh, N.J., U.S.A.) Analyzed Reagent Grade solvents were used.

Urine extraction methods. Urine from a 30-year old male was used to compare the manual and solid phase isolation methods. A first morning sample (creatinine level 311 mg/dl) was collected. Five aliquots, 3 ml each, of the urine were pipetted into test tubes. To each tube was added the internal standard, 3-chlorophenylacetic acid (0.212 mg) and hydroxylamine hydrochloride

(30 mg) and the pH was adjusted to 12 with 2 *N* NaOH. Each sample was heated at 60° for 30 min to form the oximes of the keto acids. After cooling to room temperature, the samples were divided into two sets of 5, one of which was extracted by the solid phase method and the other by the conventional manual extraction method.

Solid phase extraction. One and one-half of each aliquot was acidified to a pH below 3 with concentrated HCl and transferred directly onto a solid phase extraction tube. The organic acids were eluted with a mixture of diethyl ether—ethyl acetate (1:1, 24 ml). The extract was dried ($MgSO_4$), evaporated and transferred to a vial with ethyl acetate—methanol (1:1, 0.5 ml). The solvent was removed with a stream of nitrogen and derivatized as described below.

Manual extractions. One and one-half ml of each aliquot was acidified to a pH below 3 with concentrated HCl, and the organic acids were extracted with three 8-ml portions of diethyl ether—ethyl acetate (1:1). The combined extracts were washed and dried ($MgSO_4$) and evaporated to dryness. The extract was transferred to a vial with ethyl acetate—methanol (1:1, 0.5 ml). The solvent was removed with a stream of nitrogen and derivatized as described below.

Preparation of derivatives

The residues from above were derivatized with bis(trimethylsilyl)trifluoroacetamide (50 μl) at 60° for 30 min. Between 1 and 2 μl of this solution was used for GC—MS of the sample.

GC—MS analysis. GC and GC—MS experiments were performed on a Finnigan Model 9500 gas chromatograph, employing a 6-ft. U-shaped 1/8-in. I.D. column, packed with 10% OV-17 on 100—120 mesh Gas-Chrom Q. Mass spectra were recorded with a Finnigan model 1015 quadrupole mass spectrometer controlled by a Digital Equipment Corp. (DEC) PDP-11/20 computer. Subsequent data processing was done on a DEC PDP-11/45 with 28K words of core memory, a 5M word disc drive, teletype, printer, CRT, and Versatek printer/plotter. Each analysis was carried out by co-injecting 1—2 μl of the sample with a solution of three straight-chain hydrocarbons (with 12, 18 and 24 carbon atoms) in ethylbenzene (1 μg of each in 0.2 μl) at 70°. After 4 min The column was programmed at 4°/min and the column effluent was introduced into the MS instrument. A total of 600 mass spectra were recorded to a final temperature of 285°.

Data processing. The procedures for analyzing the raw data of the GC—MS computer system have been described previously [2]. The HISLIB program [2] was used to examine statistically the sets of GC—MS profiles collected for each extraction procedure.

RESULTS AND DISCUSSION

Solid-phase extraction tubes, which were originally developed at the Jet Propulsion Laboratory for use in the National Aeronautics and Space Administration program, are polypropylene tubes filled with an inert cellulose gauze matrix that has been purified by extensive solvent treatment [3]. The hydrophilic nature of the solid phase allows a liquid—liquid extraction separation of aqueous polar compounds from hydrophobic acids and neutrals. Advantages of

the method are the elimination of problems associated with emulsions and tube transfers. A typical urine extraction takes about 5 min whilst a comparable manual extraction takes 30 min. Since the procedure is greatly simplified, the use of a smaller starting volume (less than 0.5 ml) is possible. This is of considerable importance because urine collection from newborn babies is difficult and a number of analyses may be necessary to confirm an inborn error of metabolism.

By running 5 duplicate experiments by solid-phase and manual-extraction methods, we have established that the new technique gives comparable recoveries of the organic acids in the samples (Table I). As can be seen from the re-

TABLE I

COMPARISON OF RELATIVE CONCENTRATIONS OF ORGANIC ACIDS DETECTED AND THEIR PERCENT STANDARD DEVIATION

Acid	Manual extraction			Solid-phase extraction	
	RRI*	Relative concn.**	Percent standard deviation (n=5)	Relative concn.**	Percent standard deviation (n=5)
Glycolic	1131	42.3	21.4	64.4	15.6
3-Hydroxyisobutyric	1206	27.8	6.5	26.8	6.7
Pyruvic	1224	14.0	17.6	13.0	6.6
0-Cresol	1235	53.0	24.6	32.7	16.2
3-Hydroxyisovaleric	1244	9.0	18.7	15.4	33.9
Urea	1369	176.8	72.3	374.5	19.9
Methylfumaric	1472	11.3	24.1	13.6	15.1
Adipic	1606	8.1	11.6	7.5	20.5
Pyroglutamic	1679	28.2	30.3	38.6	20.2
5-Hydroxymethyl-furoic	1690	140.1	4.5	161.9	18.8
2-Ketoglutaric	1728	—	—	39.3	9.6
3-Hydroxyphenyl-acetic	1735	44.7	11.8	46.6	26.5
4-Hydroxyphenyl-acetic	1766	65.8	10.3	80.4	15.4
2,5-Furandicarboxylic	1806	145.0	6.4	186.2	12.9
trans-Aconitic	1858	59.4	16.5	65.7	10.2
Citric	1888	195.8	24.3	212.0	18.8
3-(3-Hydroxyphenyl)-hydracrylic	1943	269.3	9.2	321.4	20.6
Hippuric***	2118	945.3	27.3	1614.0	19.7
3-Indoleacetic	2191	13.0	33.9	28.5	22.5
Stearic	2288	6.6	21.3	—	—
3-Hydroxyhippuric	2380	320.2	9.4	389.1	12.7
4-Hydroxyhippuric	2459	23.4	11.5	32.2	15.5

*Relative retention index (on n-alkane scale).

**The internal standard, 3-chlorophenylacetic acid, has its concentration set at 100. All quantitative results are expressed in relative concentration units. Relative concentration values have not been corrected for differential extraction or detection coefficients.

***Hippuric acid elutes as a mixture of mono- and di(trimethylsilyl) derivatives. The relative concentrations of the two peaks have been combined and are reported as the mono(trimethylsilyl) derivative.

sults most metabolites are extracted with comparable efficiencies and reproducibility, and some acidic metabolites are extracted more efficiently by the solid-phase extraction method. Because elution of the extraction tube with ether—ethyl acetate could potentially add contaminants (plasticizers or nonpolar residues) from the solid phase matrix, a solvent blank was run through the tube and analyzed. When this sample was run through GC—MS and data analysis, no interfering substances were found to be present.

We conclude that solid phase extraction of organic acids from urine is simpler, faster, and less laborious than manual extraction with the same solvents. We find that most acidic metabolites are extracted with equal or improved efficiencies.

A subsequent publication will describe in detail the experimental procedures and the data processing techniques used for the analysis of organic acids.

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