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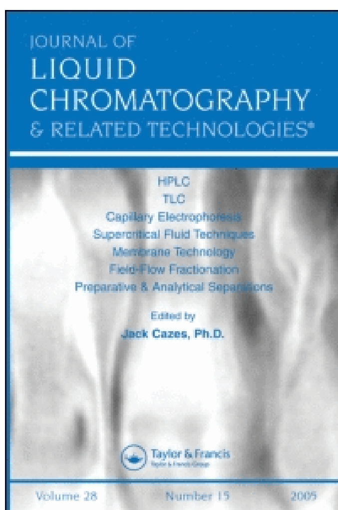

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HPLC URINARY ORGANIC ACID PROFILING: ROLE OF THE ULTRAVIOLET AND AMPEROMETRIC DETECTORS[a]

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ABSTRACT

The profiling of urinary organic acids is an important aspect of the diagnosis of inborn metabolic disorders. The carboxylic acids of interest may contain additional functional moities such as phenyl, hydroxyl, oxo, etc. The state-of-the-art method of organic acid analysis is by GC/MS. Prior to GC/MS analysis, the carboxylic acids must be isolated from urine by extraction or ion exchange chromatography and made volatile by derivatization. This is a lengthy procedure that does not lend itself to rapid analysis. We have developed a rapid procedure for the profiling of urinary α, B-unsaturated, aromatic and α-ketocarboxylic acids.

Urine containing an internal standard, 3-hydroxy-4-methoxybenzyl alcohol, is filtered through a 0.3 um Millipore filter and injected on to an HPX-87 organic acid HPLC column (Bio-Rad). The mobile phase, 4.5 mN H_2SO_4, is passed through the column at 0.8 ml/min. Detection is effected by an UV detector at 200 nm in series but upstream from an electrochemical detector with a glassy carbon working electrode at +1.15V vs. an Ag/AgCl reference electrode. At this electrode potential, phenolic, methoxyphenyl, eneolic and α-ketocarboxylic acids are oxidized and can be electrochemically detected with a glassy carbon electrode.

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Results (Rf and peak height ratio) from a urine sample are compared to a group of normal urine samples. Peaks of abnormal size and/or Rf are isolated and rechromatographed on a C_{18} HPLC column using 1% CH_3CN-0.1 M phosphate, pH 2.5. This 2-dimensional map is used in the probable identification of the peak.

INTRODUCTION

The identification and quantitation of urinary carboxylic acids is an important part in the diagnosis of metabolic disorders[1-6]. The state-of-the-art technology for the separation, identification and quantitation of urinary organic acids involves gas chromatography/mass spectrometry[1,3,5,7-10]. Gas chromatographic/mass spectrometric urinary carboxylic acid analyses are not applicable to the routine analysis of a large number of urine samples because of the long sample preparation time. What is needed is a screening process that enables one to identify rapidly a urine sample as normal or abnormal with regard to the carboxylic acids present. Abnormal urine samples could then be completely analyzed by gas chromatography/mass spectrometry. We have developed a rapid screening procedure for urinary carboxylic acids that involves neither sample extraction nor sample derivatization. This procedure is applicable to α,B-unsaturated acids, oxalic acid, phenolic acids, eneolic acids, aromatic acids and α-ketocarboxylic acids.[11]

METHODS

Equipment:

Varian 5000 HPLC with a Bio-Rad Aminex HPX-87 cationic exchange column or a Whatman PXS 10/25 ODS reverse-phase column was

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used for HPLC analysis. An Hitachi UV spectrophotometer equipped with an Altex flow cell and a Bioanalytical Systems LC-4 amperometric detector with a glassy carbon electrode were used for detection. Both detectors were connected to Linear recorders.

Materials:

3-Hydroxy-4-methoxybenzyl alcohol and all carboxylic acids were purchased either from Sigma Chemical Company or Aldrich Company and were used without further purification. Distilled and deionized water was further glass distilled from alkaline permanganate and was passed through a 0.22 um Millipore filter prior to use.

Method:

Urine Organic Acid Profile: To 0.20 ml of urine was added 25.0 ul of a 1.8 mg/ml solution of 3-hydroxy-4-methoxybenzyl alcohol. The sample was passed through a 0.3 um Millipore filter and 10 ul of the filtrate was injected on to the cation exchange column via a loop injector. The mobile phase, 4.5 mN H_2SO_4, was passed through the HPX-87 analytical column at a flow rate of 0.8 ml/min. at ambient temperature. A dual detection system employing a 200 nm ultraviolet spectrophotometric detector in series with but upstream from a glassy carbon electrode amperometric detector at +1.15V vs. an Ag/AgCl reference electrode was used. For the ultraviolet and amperometric chromatograms, each peak was assigned an Rf value and a peak height ratio relative to the internal standard.

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Eight control urine samples from normal infants from 0-6 months of age were analyzed. From the control samples, we determined an average peak height ratio for each Rf value for both the ultraviolet and amperometric chromatograms. The average peak height ratio $\pm$ SD defined normal at each Rf in each chromatogram.

Heart Cutting:[16] Metal guard columns (7.0 cm x 0.3 cm) were dry packed with Whatman CO: Pell ODS reverse-phase material. Three columns were connected to positions 1-3 on an Altex 6-way valve. The inlet to the 6-way valve was connected to the outlet from the ultraviolet detector. Each guard column was washed with 2-30 ml of the mobile phase before being used to isolate a component from the chromatogram. To isolate a specific component, the eluant from the ultraviolet detector was diverted through one of the guard columns as the component reached its maximum peak height on the ultraviolet recorder. Passage through the desired guard column was continued for 0.5 - 1.5 min. depending upon the size of the eluting component. As many as three components could be isolated per chromatographic run.

2-Dimensional HPLC Analysis: A guard column containing the isolated component was inserted into the loop injector valve as a replacement for the 10 ul loop. To the guard column was added 10 ul of a solution that consisted of 25.0 ul of 3-hydroxy-4-methoxybenzyl alcohol in 200 ul of H_2O. The contents of the guard column was injected on to an analytical Whatman Partisil PXS 10/25 ODS column. The isocratic mobile phase, 0.1 M phosphate, pH 2.5

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plus 1% CH_3CN, was pumped through the system at 1 ml/min. The components were detected at 200 nm and their Rf values calculated.

HPLC Estimation of Orotic Acid: To 0.20 ml aliquots of H_2O was added 25.0 ul of a 1.8 mg/ml 3-hydroxy-4-methoxybenzyl alcohol solution and:

a. 10.0 ul of a 5.75 umole/ml orotic acid solution.
b. 25.0 ul of a 5.75 umole/ml orotic acid solution.
c. 50.0 ul of a 5.75 umole/ml orotic acid solution.

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Each aliquot was analyzed by ion exchange HPLC using 4.5 mN H_2SO_4 at a 0.8 ml/min. flow rate. Ultraviolet detection at 200 nm was used. The orotic acid peak height/internal standard peak height ratio afforded a straight line, $y=1.68x$ ($r^2=0.99$) when graphed against the orotic acid concentration. From this working curve, the orotic acid concentration of a urine sample could be estimated from the initial 200 nm chromatogram.

Spectrophotometric Estimation of Orotic Acid: The procedure of Harris and Oberholzer[12] was used for the estimation of orotic acid in urine. The least squares working curve for this procedure was $y=0.67x$ ($r^2=1.00$).

RESULTS AND DISCUSSION

A block diagram of the HPLC system employed in the urinary organic acid screening procedure is shown in Figure 1. Because of the chemical reactions involved in the amperometric oxidation at the glassy carbon electrode, the amperometric detector must

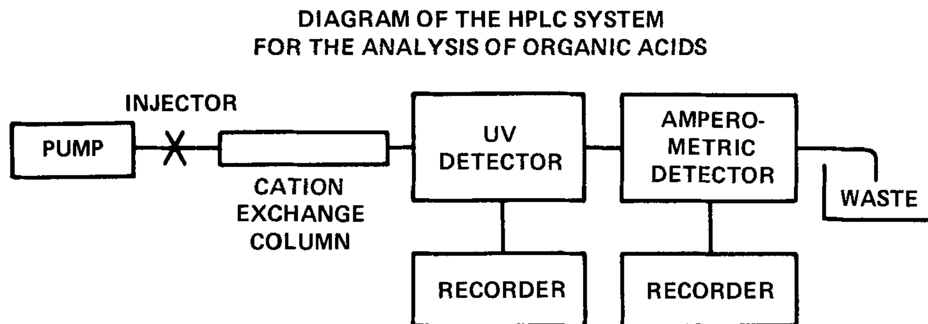

Figure 1. Block diagram of the HPLC system employed in the urinary organic acids screening.

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be downstream from the ultraviolet detector. The cation exchange HPLC column, at ambient temperature, affords adequate resolution of the urinary acids.

The standard organic acid screening procedure utilizes the UV detector at 200 nm and the amperometric detector at +1.15V vs. an Ag/AgCl reference electrode. At 200 nm, the carboxyl C=O moiety undergoes $n \rightarrow \pi^*$ absorption[13]. This transition is very weak for saturated carboxylic acids[13] ($\epsilon \simeq 50$) and is very strong for α, B-unsaturated carboxylic acids[13] ($\epsilon \simeq 10^4$). Since metabolic abnormalities generally result in urinary carboxylic acid concentrations that are 10 - 1000 times their normal value,[14,15] abnormal concentrations of saturated carboxylic acids should be detectable at 200 nm. At +1.15V, phenolic, eneolic, oxalic and α-ketocarboxylic acids are all detectable[11].

To obtain a normal urinary organic acid profile, we utilized the results from the urine of eight healthy, normal infants (0-6

months). For each urine sample, every peak in the 200 nm and +1.15V chromatogram was assigned an Rf value and a peak height/internal standard peak height ratio. The average values from these normal infants were tabulated (Table 1) and utilized as a normal urinary organic acid profile. Any peak height ratio outside the $\bar{x} \pm$ SD range was considered to be abnormal as was any peak found at an Rf value not found in our normal profile.

In Figure 2, we show the urinary profile of a critically ill two week old infant with the urea cycle defect, citrullinemia. Peak 1 (Rf 0.11) is the only abnormal component in this profile and is only abnormally large in the 200 nm ultraviolet chromatogram (peak height ratio 2.98 vs. a normal value of 0.30). Peak 1 in the +1.15V amperometric chromatogram is of a normal size (peak height ratio of 0.08 vs. a normal peak height ratio of 0.14). Based upon the Rf values of some of the known urinary organic acids (Table 2), peak 1 could be orotic acid, _cis_-aconitic acid, α-ketoglutaric acid or pyruvic acid. Orotic acid and _cis_-aconitic acid do not undergo oxidation at the glassy carbon electrode at a potential of +1.15V while both α-ketoglutaric acid and pyruvic acid do undergo oxidation. The normal peak height ratio of peak 1 in the +1.15V amperometric chromatogram eliminated α-ketoglutaric acid, pyruvic acid and any other α-ketocarboxylic acid from consideration. Both orotic acid and _cis_-aconitic acid are detectable at 230 nm and have a peak height 230 nm/peak height 200 nm ratio of 0.28. At 280 nm orotic acid has a substantial ultraviolet absorptivity while _cis_-aconitic has essential-

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TABLE 1

Normal Infant Urinary Organic Acid Profile

Rf	200 nm Ave Pk Ht Ratio ± SD	Rf	+1.15V Ave Pk Ht Ratio ± SD
0.08	2.50	0.08	0.86 ± 0.37
0.09	0.47 ± 0.14	0.09	0.17 ± 0.09
0.10	0.35 ± 0.21	0.10	0.03
0.11	0.30 ± 0.18	0.11	0.14
0.12	0.10 ± 0.06	0.12	
0.13	0.06	0.13	
0.14	0.06 ± 0.05	0.14	0.12 ± 0.13
0.16	0.48 ± 0.57	0.16	0.01
0.17	0.03	0.17	0.19 ± 0.11
0.18	0.10 ± 0.08	0.18	0.08
0.19	0.07 ± 0.07	0.19	0.06 ± 0.05
0.20	0.05 ± 0.02	0.20	0.01
0.23	0.05	0.23	
0.25	2.79 ± 1.52	0.25	1.35
0.28	0.04 ± 0.02	0.28	
0.30	0.03 ± 0.02	0.30	0.03
0.33	0.01	0.33	
0.36	0.02	0.36	0.05 ± 0.06
0.37	0.03	0.37	0.06
0.38	0.03	0.38	0.08 ± 0.06
0.39	0.03 ± 0.02	0.39	0.10 ± 0.04
0.40		0.40	0.04 ± 0.03
0.42		0.42	0.02
0.44	0.04	0.44	0.16
0.50	0.02	0.50	0.02 ± 0.02
0.54	0.02 ± 0.01	0.54	0.01
0.56		0.56	0.02
0.58	0.01	0.58	
0.60		0.60	0.02
0.66	0.02 ± 0.01	0.66	0.07 ± 0.03
0.68	0.02	0.68	
0.70	0.10	0.70	0.28
0.72	0.06 ± 0.02	0.72	0.13 ± 0.06
0.74	0.08	0.74	
0.76	0.05	0.76	
0.78	0.15	0.78	
0.81	0.20 ± 0.17	0.81	
0.86	0.04	0.86	0.04 ± 0.03
0.88	0.02	0.88	0.03

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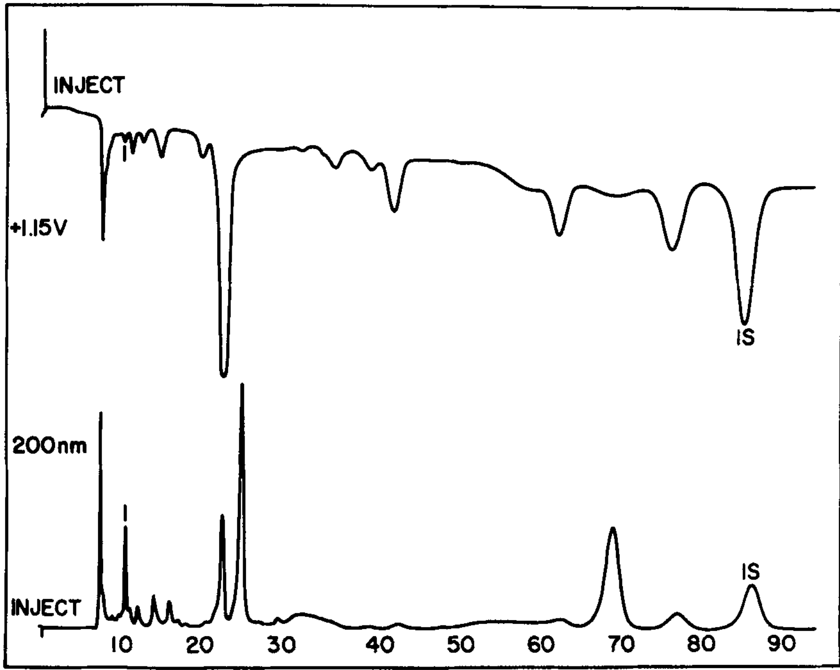

Figure 2. Amperometric (upper) and ultraviolet (lower) urinary organic acid chromatograms from a critically ill two week old infant. Internal standard (IS) is 3-hydroxy-4-methoxybenzyl alcohol. HPLC conditions are explained in the Methods section. Time scale is in minutes.

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ly no absorptivity. For orotic acid, the peak height 280 nm/peak height 200 nm ratio is 0.68 while for cis-aconitic this same ratio is 0.01. Peak 1 was found to have a peak height 280 nm/peak height 200 nm ratio of 0.75. Peak 1 was thus thought to be orotic acid. HPLC quantitation of orotic acid (see Methods) in this urine sample yielded an orotic acid concentration of 1.68 umole/ml. The spectrophotometric[12] quantitation of orotic acid in this urine sample yielded an orotic acid concentration of 1.96 umole/ml. These values

TABLE 2

Rf Value and Amperometric Activity of Standard Organic Acids

Acid	Rf	Amperometric Activity(V)
α-Keto-B-methylthiobutyric acid	0.08	+1.15
Methylcitric acid	0.09	Inactive
Oxalic acid	0.07-0.09	+0.86
Uric acid	0.09	+0.86
Orotic acid	0.11	Inactive
Pyruvic acid	0.11	+1.15
α-ketoglutaric acid	0.12	+1.15
Cis-aconitic acid	0.12	Inactive
α-ketoisovaleric acid	0.13	+1.15
α-ketobutyric acid	0.14	+1.15
Cis-oxalacetic acid	0.14	+1.15
Ascorbic acid	0.15	+0.86
α-ketovaleric acid	0.16	+1.15
α-ketocaproic acid	0.16	+1.15
α-ketoisocaproic acid	0.17	+1.15
p-Hydroxyphenylpyruvic acid	0.21	+0.86
Acetic acid	0.22	Inactive
Vanillic acid	0.25	+0.86
B-phenylpyruvic acid	0.27	+1.15
Homogentisic acid	0.37	+0.86
VMA	0.39	+0.86
3,4-dihydroxyphenylacetic acid	0.46	+0.86
p-Hydroxyphenyllactic acid	0.48	+0.86
Mandelic acid	0.48	Inactive
HMPG	0.57	+0.86
o-hydroxyphenylacetic acid	0.57-0.58	+0.86
B-methylcrotonic acid	0.62	Inactive
Catechol	0.66	+0.86
p-hydroxyphenylacetic acid	0.69	+0.86
Hippuric acid	0.81	Inactive
HVA	0.87	+0.86
Aspirin	1.16-1.20	+1.15
5-HIAA	1.46	+0.86
o-hydroxyhippuric acid	1.48	+0.86

are approximately 100 times higher than the normal orotic acid concentration in infants that are two weeks old[12].

α-Ketocarboxylic acids undergo electrochemical oxidation at the glassy carbon electrode at +1.15V but do not undergo oxidation

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at +0.86V.[11] Urine samples that have peaks of abnormal size which because of their Rf are suspected of being due to an α-ketocarboxylic acid are amperometrically profiled at 0.86V. Peak 2, Rf 0.16, in Figure 3 was present in the +1.15V chromatogram but was absent from the +0.86V chromatogram. Heart cutting[16] (see Methods) on to a 10 um C_{18} reverse phase packed mini-column effected the

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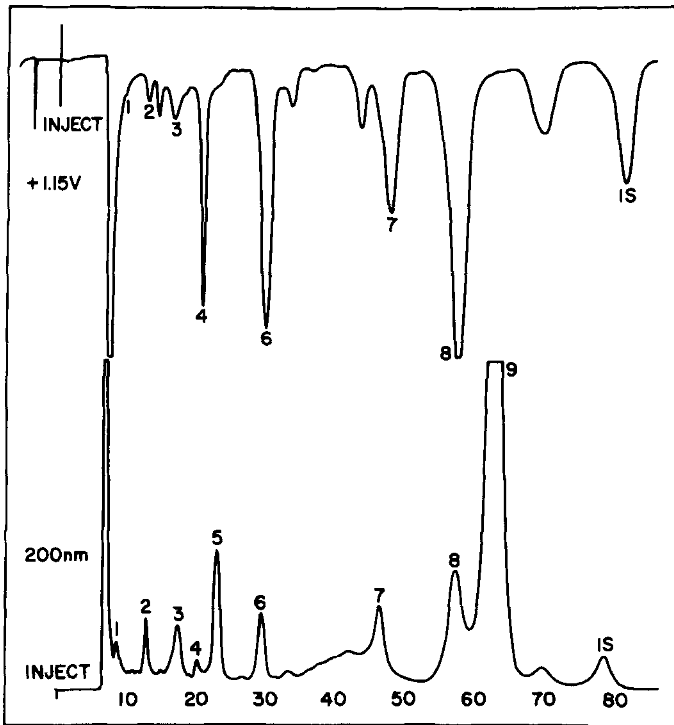

Figure 3. Amperometric (upper) and ultraviolet (lower) urinary organic acid chromatograms from a sick two year old who does not have a recognizable metabolic disease but whose urine does contain many large peaks. The numbered peaks are all larger than our standard of normal. Internal standard (IS) is 3-hydroxy-4-methoxybenzyl alcohol. Time scale is in minutes.

isolation of peak 2. Peak 2 and internal standard were rechromatographed on a 25 cm analytical C_{18} column and were detected spectrophotometrically at 200 nm. The resulting 2-dimensional map (Rf ion exchange 0.16, Rf reverse-phase 0.48) was compared with the 2-dimensional maps of authentic α-ketocarboxylic acids (Table 3). Peak 2 was thus identified as α-ketoisocaproic acid. In our system, heart cutting and 2-dimensional mapping was found to be viable only for peaks of medium to large peak heights that were well separated from neighboring components.

This method offers several advantages over the currently available procedures for identification of urinary organic acids. (1) The initial capital investment for the HPLC equipment is a small fraction of that required for a GC-MS system adequate for this usage. (2) The maintenance and supply requirements are minimal and can be performed by the operator. (3) Finally, the

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TABLE 3

2-Dimensional Maps of Some α-Ketocarboxylic Acids

Phase 1: Cation Exchange Column.
Phase 2: Reverse-phase C_{18} Column.

Compound	Rf_1	Rf_2
p-Hydroxyphenylpyruvic acid	0.21	0.42
α-Ketocaproic acid	0.16	0.54
α-Ketobutyric acid	0.14	0.20
α-Ketoisocaproic acid	0.17	0.46
α-Ketovaleric acid	0.16	0.29
Pyruvic acid	0.12	0.15
Oxalacetic acid	0.15	0.16
α-Ketoisovaleric acid	0.12	0.23
α-Ketoglutaric acid	0.11	0.17

range of inborn errors potentially detectable is quite large. Known standards have been chosen representative of a number of inborn errors including: propionic acidemia (methylcitrate); oxalosis (oxalic acid); Lesch-Nyhan syndrome (uric acid); urea cycle enzymopathies (orotic acid); lactic acidoses (pyruvic acid); branched chain ketoaciduria (α-ketoisocaproic; α-ketomethylvaleric; α-ketoisovaleric); phenylketonuria (phenylpyruvic); tyrosinemia (p-hydroxyphenylactic); alkaptonuria (homogentisic); B-methylcrotonyl C_OA carboxylase deficiency (B-methylcrotonic acid). Further refinement in chromatographic procedure should result in extension of this method to the detection of metabolites characteristic of other inborn errors.

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