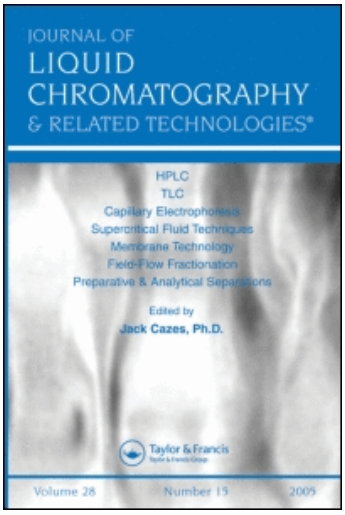

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HIGH RESOLUTION SEPARATION OF URINARY ORGANIC ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Reverse phase, anion exchange, and two-dimensional HPLC techniques were studied in order to increase resolution of organic urinary acids for eventual quantitative measurements. Reverse phase HPLC with a phosphate buffer/acetonitrile gradient yielded a separation of over 85 components in forty minutes and a peak area reproducibility of better than 5%. Connecting two reverse phase columns together resulted in the separation of 110 components. Anion exchange chromatography was determined to be of little use in resolving urinary acids in a resonable time except as the first stage in two-dimensional chromatography where fractions from the anion exchange column were injected into a reverse phase column. Over 139 components were separated by this two-dimensional method.

INTRODUCTION

Separation of the components present in complex mixtures, such as urine and other biological fluids, presents unique chromatographic problems because of the large variety and number of compounds in these samples. One such group of components, the low molecular weight organic acids found in human urine, is of particular interest since it includes intermediates and end products of various metabolic pathways which, in turn, can be affected by diseases (3,4). In most cases, correlations between the presence of a specific disease and urinary acids are related to concentration variations rather than to the presence or

absence of a specific component (5,6). For diseases which have not been thoroughly investigated, it is much easier to first separate all of the components in the sample and then to identify and quantify those which show variations compared to healthy individuals than it is to isolate and identify all of the components. This former technique has been termed "quantitative metabolic profiling (6)". To be successful this technique requires highly efficient chromatographic methods capable of completely resolving all of the components in the sample in order to facilitate peak integration.

Anion exchange chromatography was one of the first methods to be used in separating urinary components; however, in order to separate 100 of more constituents, long columns and separation times in excess of 20 hours were necessary (7,8,9). More recently, over 130 urinary acids were separated by gas chromatography (GC) (10) and over 230 acids have been separated by capillary GC (11). Although they are very efficient and allow coupling to a mass spectrometer for identification, GC methods are limited to components of high volatility; otherwise, derivatization steps are required which may decrease recovery of the components. High performance liquid chromatography (HPLC) is not limited by component volatility nor by the presence of various salts which often accompany the extraction procedure. Molnár and Horváth recently reported the separation of over 100 urinary organic acids in 30 minutes by reverse phase HPLC (12). However, many of the peaks were not sufficiently resolved to be useful for quantitative metabolic profiling.

Two-dimensional chromatography is a technique in which fractions from one chromatographic mode are transferred to another chromatographic mode to increase resolution (13). Some applications of this technique have been reported for separating urinary acids (14,15), most notably by Spiteller and Spiteller who separated about 500 acid components using a two-dimensional system employing thin layer chromatography and then capillary GC-mass spectrometry (16).

This paper describes some refinements on the reverse phase HPLC method and also presents the results of a two-dimensional study employing anion exchange and then reverse phase HPLC for the separation of urinary organic acids.

MATERIALS

Apparatus

An Altex 322 MP microprocessor controlled gradient HPLC (Beckman Instrument Co., Berkeley, CA) equipped with two model 100A pumps, a model 400-12 mixer, a model 210 injection valve with a 20 µL sample loop, a model 100-10 Hitachi variable VIS-UV wavelength detector set at 280 nm, and a Linear model 385 chart recorder were used throughout this study. Peak areas were determined by a Hewlett Packard model 3380-S electronic integrator.

Columns

Analytical columns were packed by the manufacturer; they included: RSiL-AN 5 µm anion exchange, 25 cm x 4.6 mm I.D. (Alltech Associates, Deerfield, IL), SynChropak AX300, 10 µm anion exchange, 25 cm x 4.1 mm I.D. (SynChrom, Inc., Linden, IN), and Beckman Ultrasphere ODS, 5 µm reverse phase, 25 cm x 4.6 mm I.D. Guard columns consisting of either the RSiL-An anion exchange material (5.0 cm x 4.6 mm I.D.) or Lichrosorb RP-18, 10 µm (E. M. Merck) (7.0 cm x 2.2 mm I.D.) were packed in this laboratory with a model 704 Micromeritics slurry packer (Micromeritics, Norcross, GA). No guard column was used with the SynChropak column. The guard and analytical columns were kept at the appropriate temperature controlled by a Thermomix Model 1460 constant temperature water bath.

METHODS

Sample Preparations

Urine samples were collected from an apparently healthy adult male, combined, then frozen in 30 mL vials at -80°C until ready for use. The acids were extracted from urine either by concentra-

tion on a DEAE Sephadex A-125-120 (Sigma Chemical Co., St. Louis, MO) anion exchange column followed by elution with pyridinium acetate and then lyophilization to dryness (for anion exchange and reverse phase chromatography studies) or by extraction into an ether-ethyl acetate mixture followed by evaporation of the organic layer (for two-dimensional chromatography studies). Details of these procedures are given by Rehman et al. (17).

HPLC

Aqueous buffers were prepared either from reagent grade potassium dihydrogen phosphate (Mallincrodt, St. Louis, MO) with the pH adjusted using 85% orthophosphoric acid (Fisher Scientific Co., Fair Lawn, NJ) or from glacial acetic acid with the pH adjusted using sodium hydroxide. Water was purified on a Milli-Q system (Millipore Corp.). Buffers and HPLC grade solvents (acetonitrile and methanol, Burdick and Jackson Laboratories, Muskegon, MI) were filtered through a 1.2 μm (aqueous solvents) or 0.5 μm (organic solvents) filter and then deaerated before using.

For ion exchange chromatography a concave gradient was used starting with either 0.02 M pH 3.0 phosphate buffer or 0.10 M pH 7.0 acetate buffer and increasing to 1.13 M phosphate or 4.0 M acetate, respectively, followed by a reverse gradient to the initial conditions. The column was equilibrated at the initial conditions for fifteen minutes before injecting another sample. The flow rate was 1.0 mL/min.

For reverse phase chromatography the initial mobile phase was the appropriate aqueous buffer. The acetonitrile or methanol concentration was increased to 39% in 36 minutes in a concave fashion, then linearly to 70% in 15 minutes (to insure complete elution of all components), followed by a reverse gradient and equilibration under initial conditions for 15 minutes (Figure 1). The flow rate was maintained at 2.0 mL/min.

In the studies involving two-dimensional chromatography, fractions were taken from the RSiL-AN anion exchange column, con-

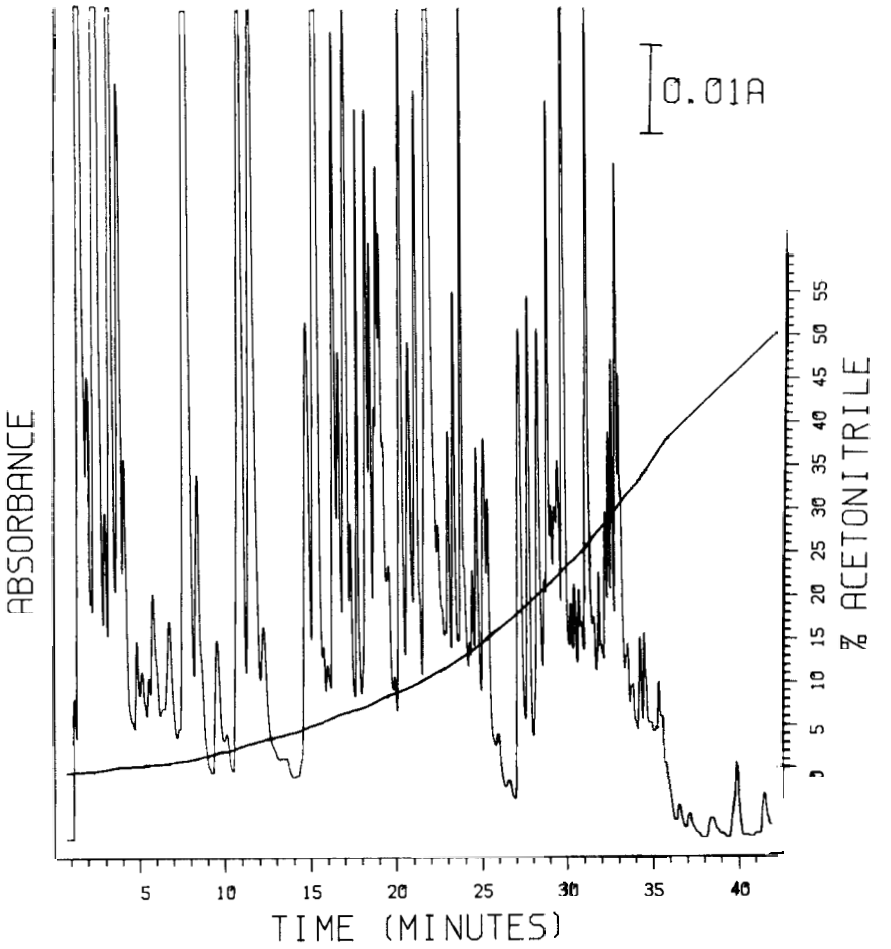


FIGURE 1. Separation of urinary acids on a reverse phase column using a concave pH 2.1 0.2M phosphate buffer/ acetonitrile gradient.

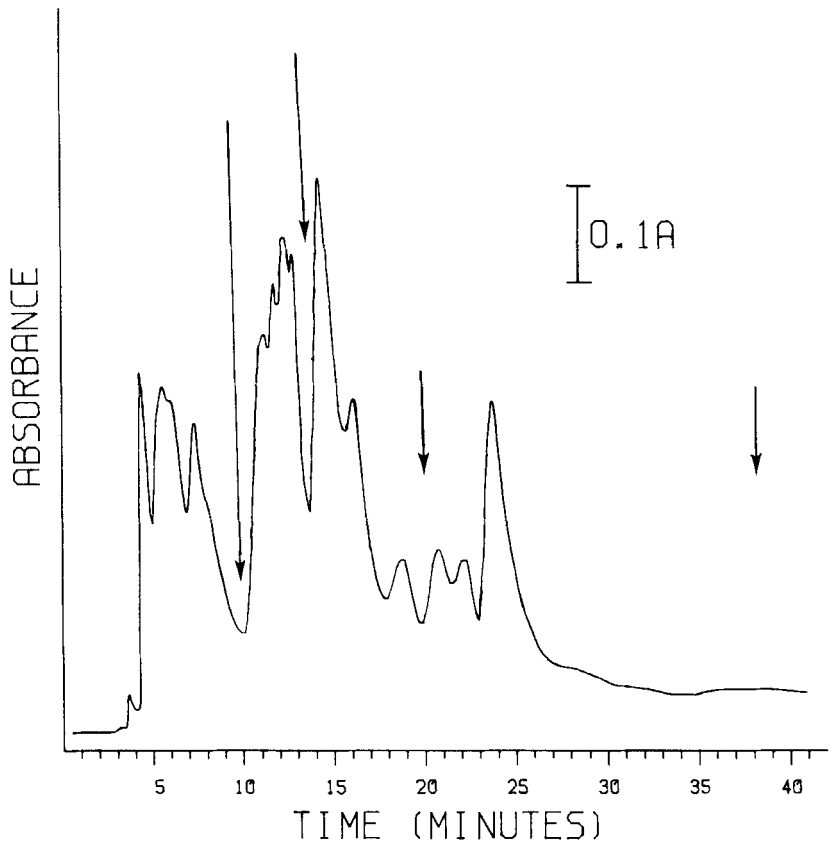


FIGURE 2. Separation of urinary acids on an anion exchange column using a linear gradient from 0.05 M phosphate buffer at pH 3.0 to 0.5 M phosphate in 50 minutes. Fractions were taken at the times indicated by arrows.

centrated, then injected into the reverse phase column, as follows. For the anion exchange separation, 100 µL of sample was injected, and a linear gradient from 0.05 M phosphate buffer (pH 3.0) to 0.5 M phosphate in 50 minutes was employed. Four fractions were collected without stopping the flow (Figure 2). The fractions

were extracted into ether-ethyl acetate, the organic layer evaporated, and the residue dissolved in 50 μL of 0.20 M (pH 2.1) phosphate buffer. A twenty μL aliquot of each fraction was then injected into the reverse phase column using the same conditions given above.

RESULTS AND DISCUSSION

Ion Exchange Chromatography

Two types of anion exchange column packings were investigated: RSiL-AN using an aqueous pH 3.0 phosphate buffer gradient with increasing phosphate concentration at 60°C and SynChropak AX300 using an aqueous pH 7.0 acetate buffer gradient with increasing acetate concentration at 30°C. Since most of the urinary acids in the extract have pK's between 4 and 5 (18), the predominate mode of separation at pH 3.0 is probably sorption rather than ion-exchange (19); ion exchange would predominate at pH 7.0 or above. Neither column gave a good separation of urinary acids; only approximately 25 resolved or partially resolved peaks were observed for both columns. Although the peaks from the SynChropak column were sharper, the RSiL-AN column and phosphate mobile phase were chosen for the studies involving two-dimensional chromatography because the pH was more compatible with that of the aqueous phase for the reverse phase column (see below).

Reverse Phase Chromatography

Preliminary studies with standard acid solutions and urinary acid extracts indicated that the sharpest, most symmetrical peaks and the best separation of urinary acids on an Ultrasphere column are obtained with a pH 2.1 phosphate buffer (0.2 M) at 50°C using a concave gradient from 0 to 70% acetonitrile. Under these conditions, approximately 85 resolved and partially resolved peaks were obtained (Figure 1). These results are comparable to those of Molnár and Horváth (12), who reported 104 peaks under similar conditions using a 5 μm Lichrosorb RP-18 column.

The peak area reproducibility of the method was determined by three injections of the same urine extract. Twenty-eight of

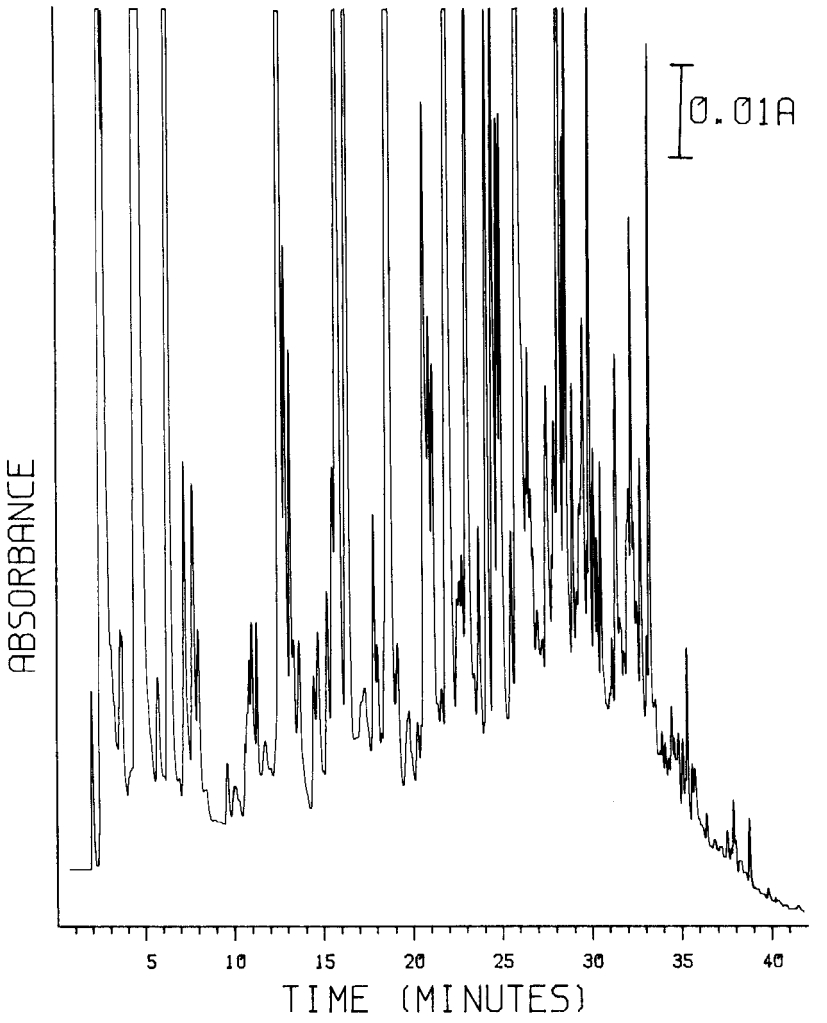


FIGURE 3. Separation of urinary acids on two reverse phase columns connected together. The gradient is shown on Figure 1.

the larger and better resolved peaks were chosen for comparison. The mean and median percent standard deviations of each peak for the raw areas were 5.8 and 4.4, respectively and 5.0 and 3.5, respectively for normalized areas.

The peak retention times generally had standard deviations from 0.07 to 0.18 minutes, although somewhat larger deviations were sometimes observed for peaks having retention times between 5 and 12 minutes.

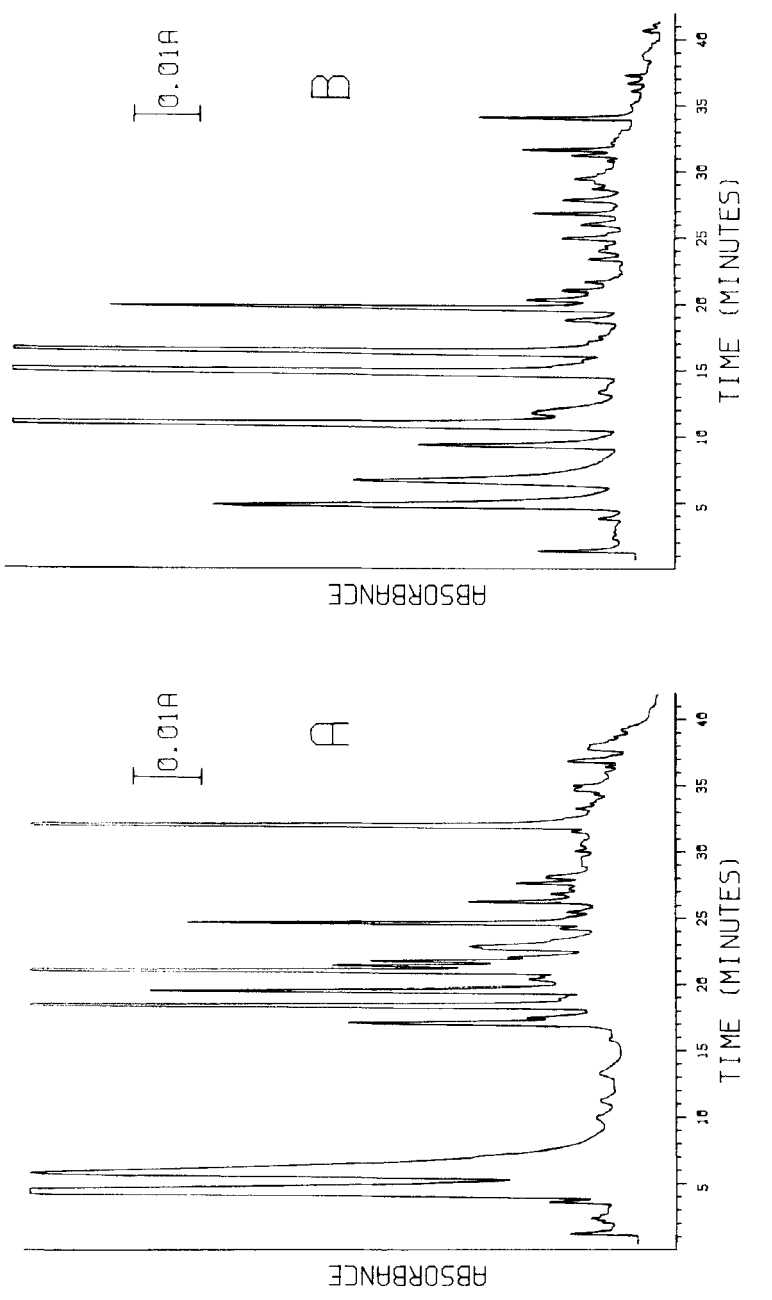
A similar separation and number of peaks (although a different peak pattern) were observed when methanol was substituted for acetonitrile in the gradient; however, the pressure reached 5000 psi compared to 3500 psi with acetonitrile. Likewise, a similar chromatogram was obtained when 0.1 M acetic acid (pH 2.9) was substituted for phosphate. Although not a buffer, acetic acid may be appropriate for use when isolation of separate peaks or fractions from the reverse phase column is required, since acetic acid can be removed by lyophilization.

As stated above, in order for quantitative metabolic profiling to be useful, it is necessary for all of the components in the sample to be as completely resolved as possible. While the optimum conditions for the Ultrasphere column probably have been attained, it is apparent from Figure 1 that many of the peaks remain unresolved.

One means of increasing resolution is to increase column length by connecting two or more columns together. Under identical conditions as with one column, connecting two and three columns together produced nearly 110 (Figure 3) and 90 peaks respectively. The apparent decrease in resolution with the three column system is probably due to the increase in interconnective tubing. In addition, using three columns resulted in an extremely high pressure (8000 psi).

Two-Dimensional Chromatography

A urine extract was separated first on the RSiL-AN anion exchange column at pH 3.0. Four fractions were taken during the



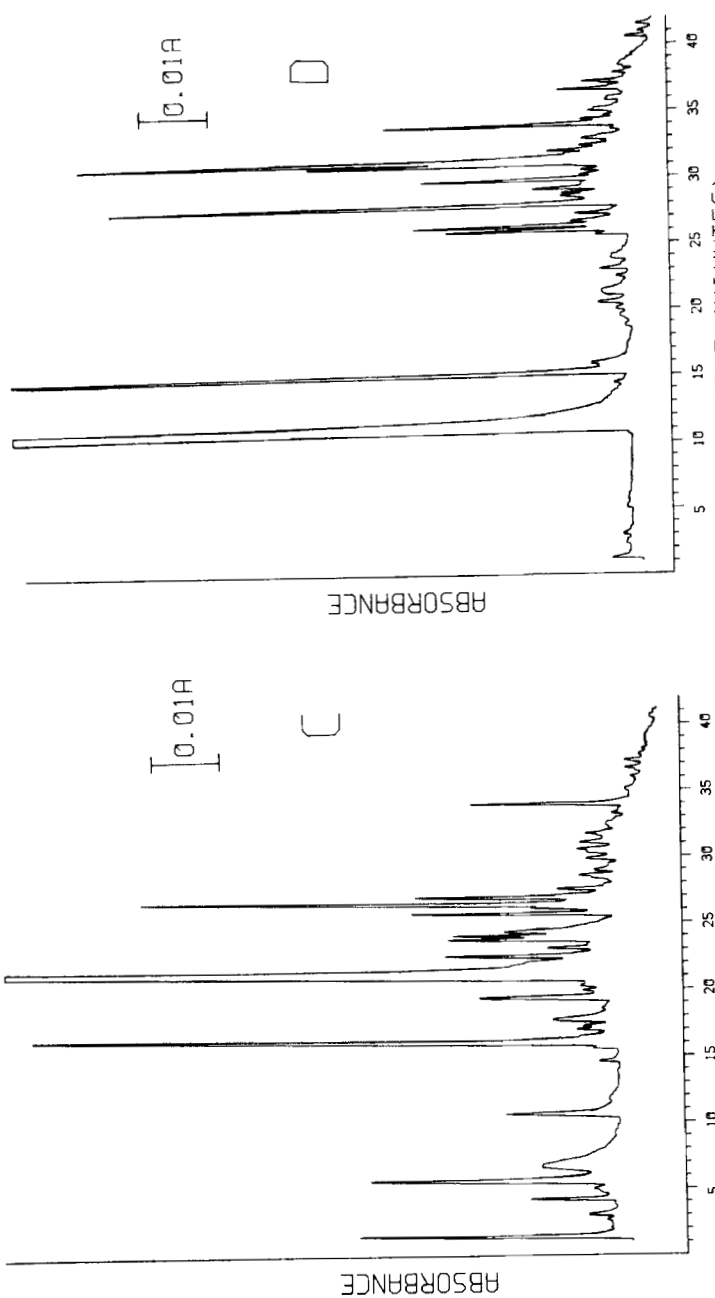


FIGURE 4. Separation of fractions collected from the anion exchange column (as indicated in Figure 2) on a reverse phase column. The gradient is shown in Figure 1. A. Fraction 1. B. Fraction 2. C. Fraction 3. D. Fraction 4.

separation (indicated by the arrows in Figure 2). The acids were extracted, concentrated, and then injected into the Ultrasphere reverse phase column. Fractions one to four yielded 55, 46, 48, and 56 peaks, respectively (Figure 4A-D). Considering overlap from adjacent fractions, of the 205 total peaks, 139 appear to be due to different components.

Identification of Components

When the quantitative metabolic profiling approach is used to investigate diseases, it is not necessary to identify every

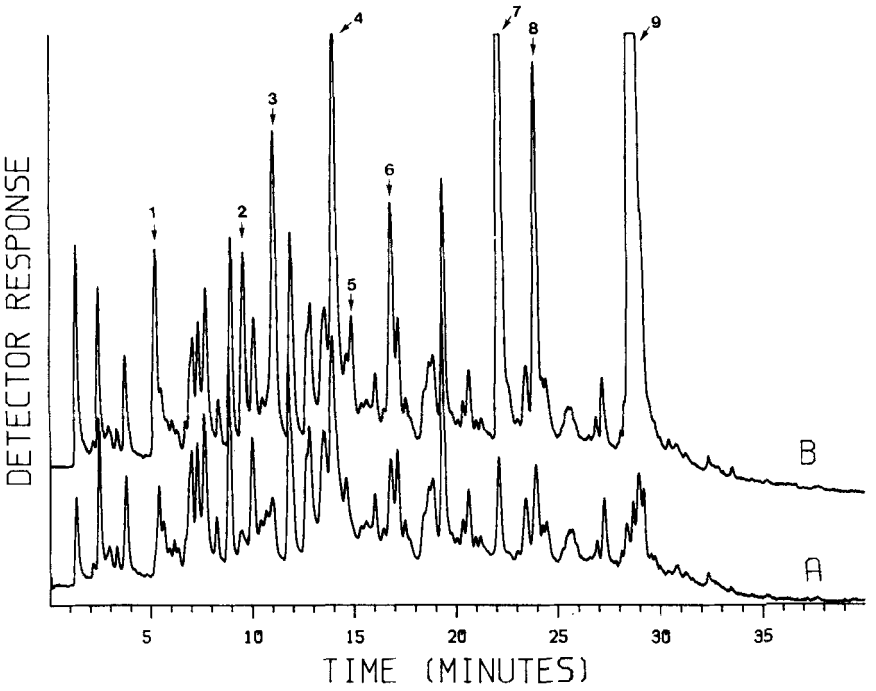


FIGURE 5. A. Chromatogram of a urinary acid extract diluted 1/1 with pH 2.1 phosphate buffer.
B. Chromatogram of the extract from Figure 5A but diluted 1/1 with a solution containing nine organic acids at 100ppm each. The identity of each numbered peak is given in Table 1. Both chromatograms were obtained using the conditions given in Figure 1 and in the text.

TABLE 1

Identification of Some Organic Acids Present in Urine

Peak Number*	Compound
1	4-hydroxy-3-methoxymandelic
2	3,4-dihydroxyphenylacetic acid
3	p-hydroxybenzoic acid
4	m-hydroxybenzoic acid
5	m-hydroxyphenylacetic acid
6	4-hydroxy-3-methylphenylacetic acid
7	4-hydroxy-3-methoxycinnamic acid
8	indole-3-acetic acid
9	transcinnamic acid

*Refers to those peaks which are numbered in Figure 5

component in the chromatogram. Only those components need be identified which show statistically significant differences between samples taken from diseased *versus* normal subjects while positive identification of these components can be made by retention time comparison and/or by spiking the sample with known compounds. This is shown in Figure 5 which is a comparison of chromatograms of a urinary organic acid extract *vs* the same extract spiked with nine organic acids which are commonly found in urine (10). Table 1 lists each peak number and its identity. Clearly, this tentative peak identification is facilitated by a highly efficient and reproducible HPLC separation.

CONCLUSIONS

Reverse phase HPLC yields a rapid and reproducible separation of urinary acids; however, in order to increase resolution for quantitative measurements, more efficient columns are required. Efficiency may be increased by connecting columns together; however, it is somewhat limited by interconnective tubing and increase operating pressure. As more efficient ODS

columns are developed, especially microbore columns, a greater resolution may eventually be realized.

Although tedious and time consuming, two-dimensional chromatography results in a separation of the largest number of urinary acids yet reported by HPLC. In addition, a greater degree of baseline separation is achieved over one dimensional HPLC since fewer components are present in each fraction than are present in the entire sample. Its use is suggested if components of a specific fraction are to be identified and/or quantified. With the development of on-line techniques; i.e. automatic column switching, this method may eventually become more practical.

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