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PHOTODIODE ARRAY ULTRAVIOLET SPECTROPHOTOMETRIC PROFILING OF CARBOXYLIC ACIDS IN PHYSIOLOGICAL FLUIDS

D.N. BUCHANAN* and J.G. THOENE

University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

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SUMMARY

In this paper the use of a computer-controlled photodiode array spectrophotometric detector for the high-performance liquid chromatographic (HPLC) profiling of carboxylic acids in physiological fluids is reported. The ultraviolet spectrum of the flowing eluent is obtained at 6-sec intervals and is displayed as the absorbance at 190 nm. A three-dimensional (time versus wavelength versus absorbance) presentation of the HPLC profile facilitates peak identification through ultraviolet spectrum matching and relative retention time comparison with carboxylic acid standards. Several examples of HPLC urinary carboxylic acid profiles from infants with various inborn errors of metabolism are shown.

INTRODUCTION

The high-performance liquid chromatographic (HPLC) profiling of carboxylic acids in physiological fluids is a means of distinguishing normal and abnormal specimens as a first approach to the detection of inborn errors of metabolism [1,2]. The on-column injection of filtered raw urine or plasma to which has been added an internal standard results in fast analyses with minimal sample preparation. The major drawback to this procedure is the lack of a means of peak identification. Both the single- [2] and the dual-column [1] profiling techniques result in most of the carboxylic acids eluting in the first 6—18 min. Thus, most of the acids of interest have similar elution volumes and are hard to identify by peak augmentation. Wavelength ratioing may facilitate peak identification but generally requires one or more additional passes through the HPLC system. Use of a computer-controlled photodiode array spectrophotometer as an HPLC detector affords the UV spectra of each peak as it elutes. Through the use of appropriate software (see Experimental), the UV spectrum and its 1st and 2nd derivatives [3] can be obtained for any peak or portion of a peak, i.e. leading edge, apex or trailing edge. In addition, a three-

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dimensional (time versus wavelength versus absorbance) representation of the chromatogram can be produced. This facilitates peak identification and the determination of peak purity. In this paper we report results obtained using a computer-controlled photodiode array spectrophotometer as a detector for the dual-column HPLC profiling of carboxylic acids in urine. Peak identity was in some cases confirmed by fraction collecting and gas chromatographic—mass spectrometric (GC—MS) analysis of each fraction.

EXPERIMENTAL

Chemicals

All carboxylic acids were either from Aldrich (Milwaukee, WI, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.) and were used as received. HPLC-grade water was from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Equipment

A Varian 5000 high-performance liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) with an HP 1040 A photodiode array spectrophotometric detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) interfaced with an HP 85 computer with a dual-disk drive was used for all determinations. An HP 4770A plotter was used for all chromatograms and spectral plots. An HP 5995 B gas chromatograph—mass spectrometer was used for all MS determinations with a 5% OV-22 glass column (1.83 m × $^1/_8$" I.D.) (Supelco, Bellefonte, PA, U.S.A.) for all GC separations.

Procedure

Sample preparation and chromatographic conditions have been previously reported [1] and consisted of an HPX 87H organic acid column (Bio-Rad Labs., Richmond, CA, U.S.A.) in series with a 15-cm, 5 μm particle size, ODS column (Bio-Rad Labs.) with a mobile phase of sulfuric acid pH 2.5. The HP 85 data disk was formated to accept 1000 records while the HP 1040 A detector was set to detect and save all spectra from 190—350 nm at a 0.80 min peak width. The monitor wavelength was 190 nm and the attenuation was 0.2 a.u.f.s. for a 10-μl injection.

Fraction collection was accomplished by injecting either 0.50 ml or 1.00 ml of urine while the detector was set at 2.5 a.u.f.s. The volume injected depended upon the creatinine concentration of the urine sample. Urine samples with a creatinine concentration from 0.25 to 1.00 mg/ml required a 1.00-ml aliquot to be injected onto the HPLC columns while samples with a creatinine concentration >1.00 mg/ml required a 0.50-ml aliquot. Fraction collection and GC—MS analysis of samples having a creatinine concentration < 0.20 mg/ml were unsuccessful. During the first part of the run, fractions were collected at 1-min intervals (6—22 min) while 3-min intervals were used for the duration of the run (23—75 min). Each fraction was rechromatographed (10 μl injected; 0.1 a.u.f.s., 0.80 min peak width) to determine the number of components present. A three-dimensional profile (elution time versus wavelength versus absorbance) and the UV spectrum and its 1st and 2nd derivative spectra were produced for each peak in each fraction. Each fraction was saturated with sodium

chloride and extracted twice with 3 ml of ethyl acetate and once with 3 ml of diethyl ether. The combined organic extracts were taken to dryness under a stream of air and converted to the trimethylsilyl (TMS) derivative by the addition of 30 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and heating for 15 min at 90°C. A 1-μl sample was injected into the GC—MS system.

The GC parameters were: flow-rate: 25 ml/min; injector temperature: 250°C; initial temperature: 90°C; temperature hold: 4 min; final temperature: 280°C; rate of heating: 5°C/min; final temperature hold: 15 min. An electron-impact (EI) mass spectrum was taken for each peak and identification was by comparison of spectra from known standards and from published spectra of TMS carboxylic acid derivatives [4].

RESULTS AND DISCUSSION

A chromatogram for an abnormal urine sample is shown in Fig. 1. Most of the peaks are found within the first 22 min of the run. Abnormally large peaks are observed at 8, 12 and 14 min but their identity cannot be ascertained by reference to the relative retention time (RRT) alone. Using the three-dimensional display capability (Fig. 2A—C) of the software, we can both separate the peaks by varying the time axis and tentatively identify some of them. The peaks at 12 and 14 min are thought to be lactic acid and succinic acid, respectively, based on their UV spectra. Lactic acid is frequently detected in all patients while the succinic acid is probably of bacterial origin [4].

The peak at 8.2 min has the correct RRT and UV spectrum for orotic acid. Orotic acid is normally a minor component of urine but is markedly elevated

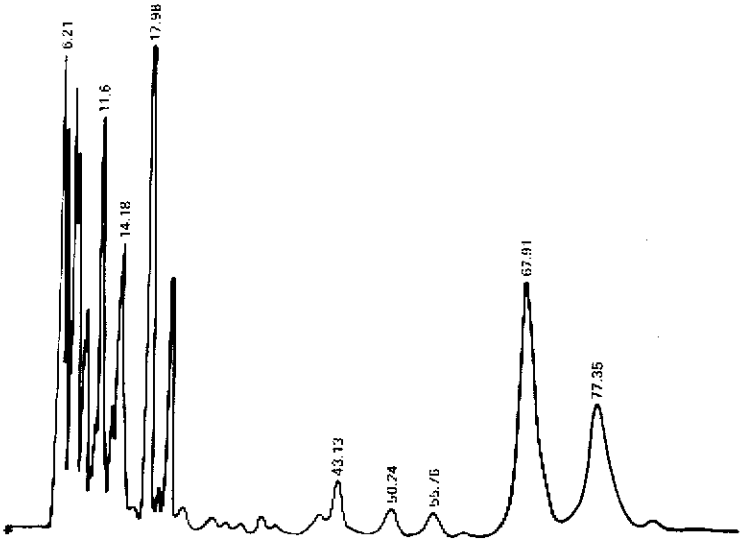
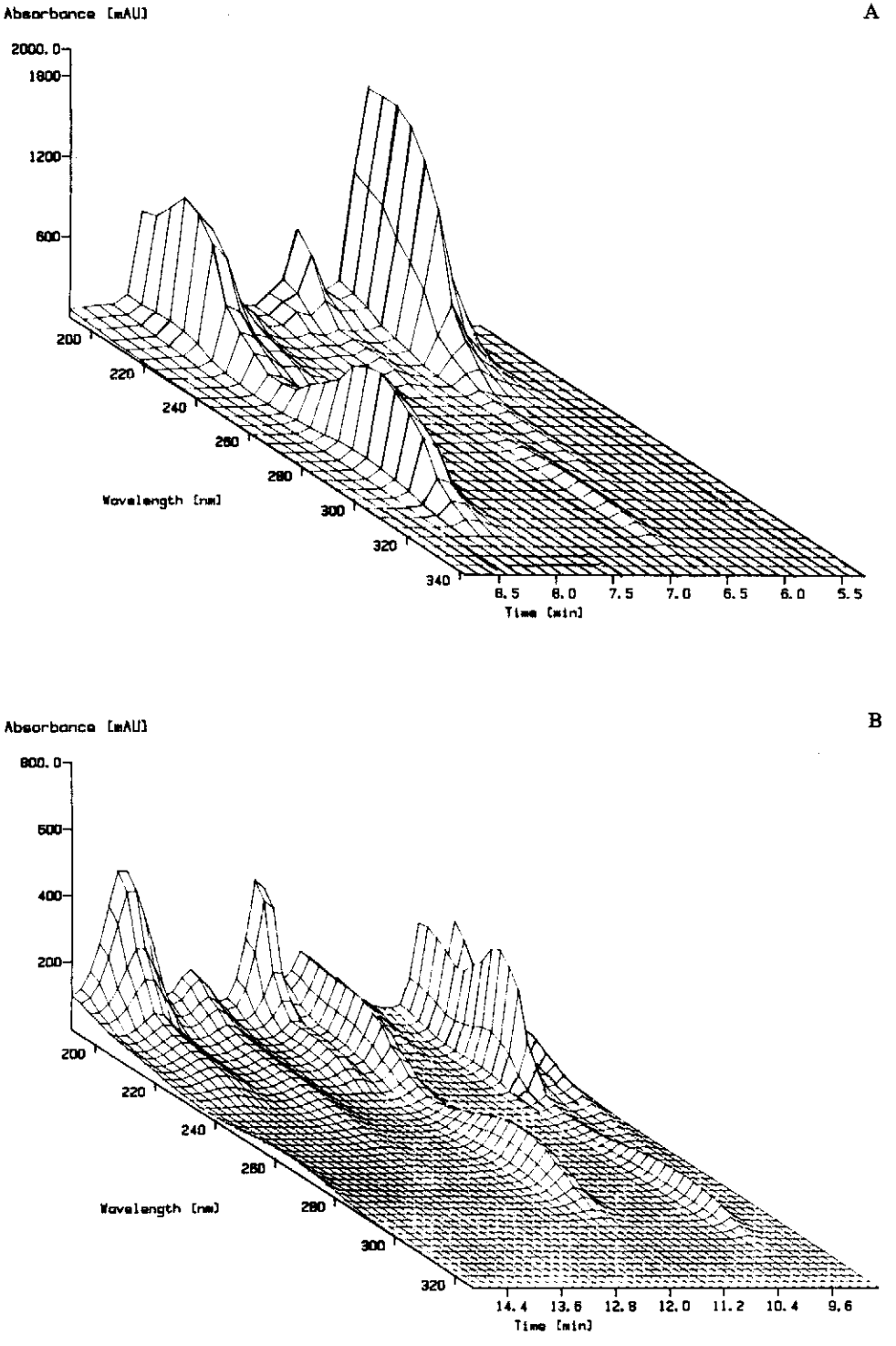


Fig. 1. HPLC carboxylic acid profile from a 10-μl injection of urine; 190 nm; 0.200 a.u.f.s.; sulfuric acid pH 2.5 at 0.8 ml/min. The peak at 17.98 min is uric acid and the peak at 67.91 min is hippuric acid plus 4-hydroxyphenylacetic acid.


A
Absorbance [mAU]
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1800
1200
600
200
220
240
260
280
300
320
340
Wavelength [nm]
6.5
8.0
7.5
7.0
6.5
6.0
5.5
Time [min]
B
Absorbance [mAU]
800.0
600
400
200
200
220
240
260
280
300
320
Wavelength [nm]
14.4
13.6
12.8
12.0
11.2
10.4
9.6
Time [min]

Fig. 2.

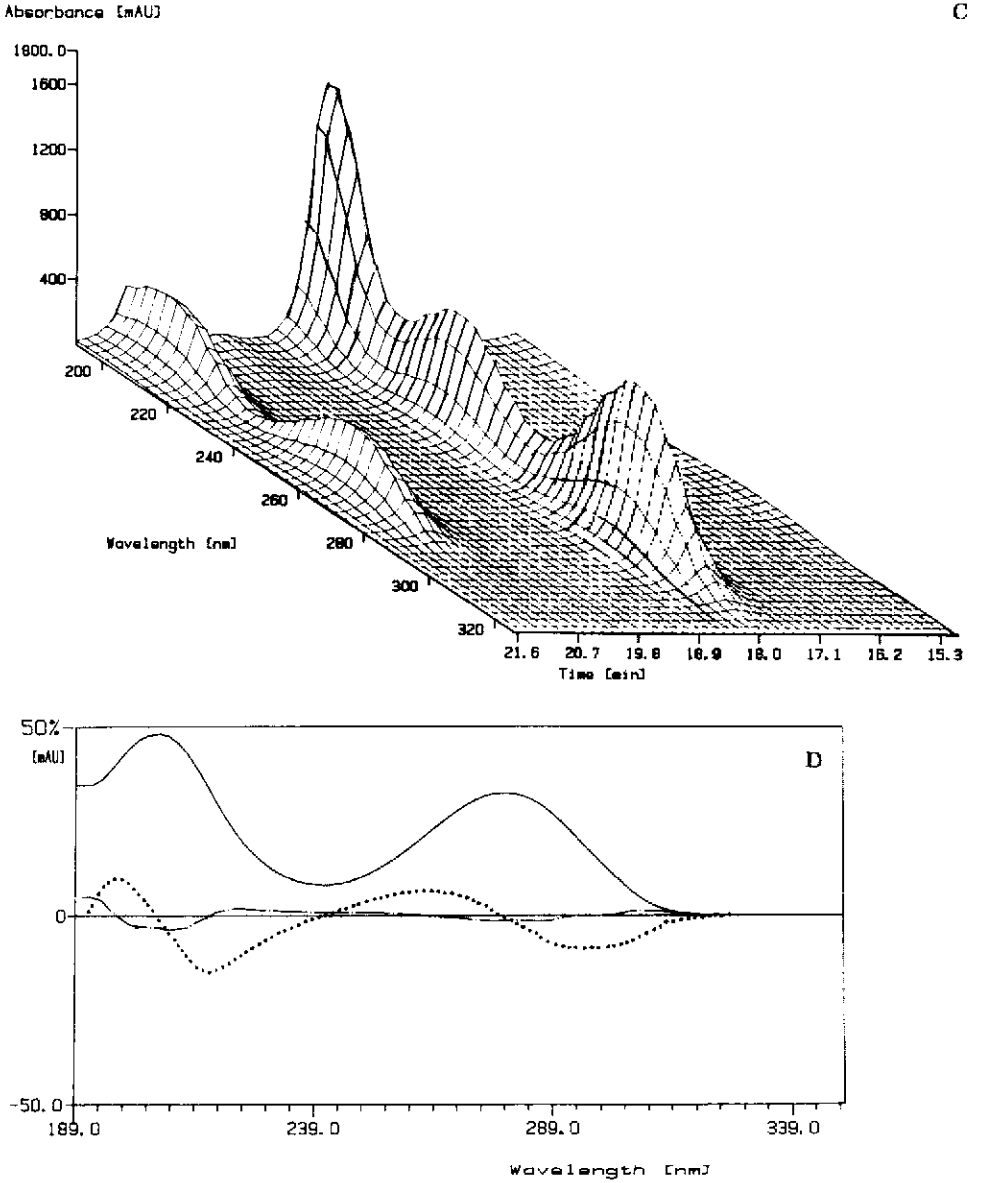

Fig. 2. Three-dimensional profile of the urine sample in Fig. 1. (A) The 5—9 min interval of the chromatogram; (B) the 9—15 min interval of the chromatogram; (C) the 15—22 min interval of the chromatogram shown in a reversed time scale so that the peak at 21 min (uracil) can be seen; (D) the 0th, 1st and 2nd derivative spectra of authentic orotic acid.

in urea cycle defects [5]. The UV spectrum of the 8.2-min peak and its 1st and 2nd derivative spectra (Fig. 2D) match that of authentic orotic acid. Other components identified in the chromatogram include the 17.98-min peak which was shown by its UV spectrum to be uric acid [1,2] and the 67.91-min peak which was thought to be hippuric acid by comparison of its UV spectra with that of authentic hippuric acid.

TABLE I

IDENTIFICATION OF SOME CARBOXYLIC ACIDS FROM URINE

Retention time (min)	Relative retention time*	Identity	Means of identification
6.21	0.07	Oxalic acid	GC—MS
7.92	0.09	Orotic acid	UV
9.39	0.11	Citric acid	GC—MS
		Isocitric acid	GC—MS
11.60	0.14	Lactic acid	UV and GC—MS
		3-Hydroxypropanoic acid	GC—MS
		Succinic acid	GC—MS
14.18	0.17	Succinic acid	GC—MS
17.98	0.21	Uric acid	UV
		Glutaric acid	GC—MS
		Pyroglutamic acid	GC—MS
20.93	0.25	Uracil	UV and GC—MS
43.13	0.51	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid	GC—MS
50.24	0.59	3- or 4-Hydroxyhippuric acid	GC—MS
67.91	0.80	Hippuric acid	UV and GC—MS
		4-Hydroxyphenylacetic acid	GC—MS

*Internal standard (3-hydroxy-4-methoxybenzyl alcohol), RRT = 1.00.

Usually, the UV spectrum of a peak does not exactly match that of any of the standard acids which elute at the same RRT. This was thought to be due to the simultaneous elution of two or more compounds. In order to confirm this, we collected fractions at 1-min intervals for the first 22 min of the profile and at 3-min intervals for the duration of the run. Each fraction was converted to the TMS derivative and analyzed by GC—MS. Our initial attempt was unsuccessful. However, when a larger volume of urine was analyzed (0.5—1.0 ml), fraction collection and derivatization were successful. The injection volume necessary for fraction collection was dependent upon the creatinine concentration of the urine (see Experimental). Table I shows the results of fraction collection. As would be expected, there is some overlap between fractions. Since each fraction must be extracted and derivatized, it was expected that not every peak (irrespective of its size in the 190-nm chromatogram) could be identified by GC—MS analysis. The orotic acid and uric acid peaks are good examples since they both have strong absorptivity at 190 nm but were not identified by GC—MS analysis while the uracil peak (20.9 min) was identified by GC—MS. Perhaps the use of a non-extractive isolation technique such as silicic acid absorption [6] or Extrelut columns [7] and the use of a capillary GC column might improve the GC—MS analysis of collected fractions.

Coeluting compounds may not be detectable by UV analysis because of the vast differences in their molar absorptivities. Glutaric acid and pyroglutamic acid were found to coelute with uric acid (Table I) but were not observed in the UV spectrum of this peak. Coeluting compounds may also escape UV detection when they have the same or similar UV spectrum. The 67.91-min

peak of Fig. 1 has the expected RRT and UV spectrum for either hippuric acid or 4-hydroxyphenylacetic acid and cannot be positively identified by its UV spectrum. GC—MS analysis of this peak (Table I) showed that both compounds were present.

Fig. 3A shows the 190-nm carboxylic acid profile of a urine sample from an infant with methylmalonic aciduria. The peak at 11.83 min is larger than

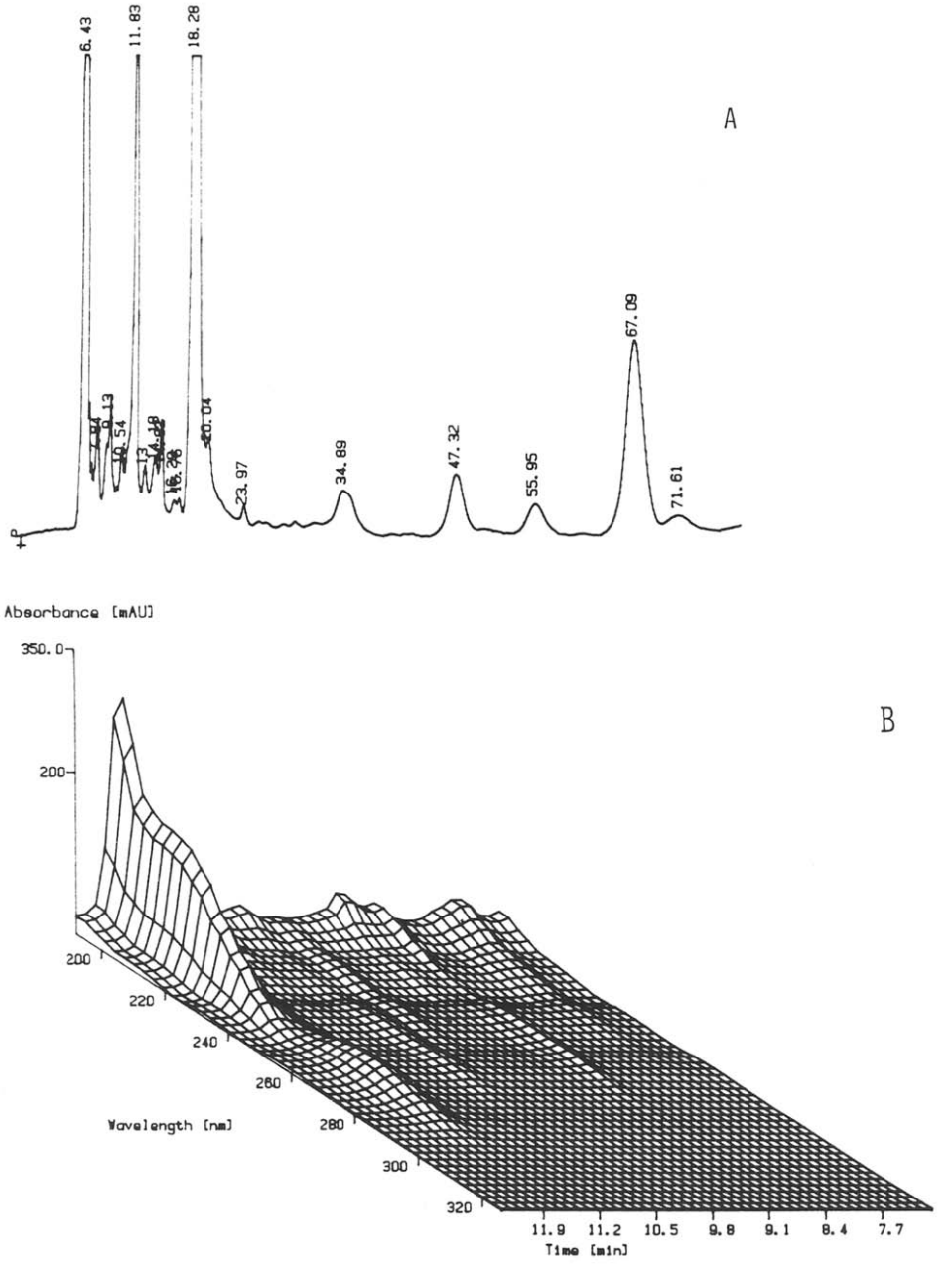

Fig. 3.

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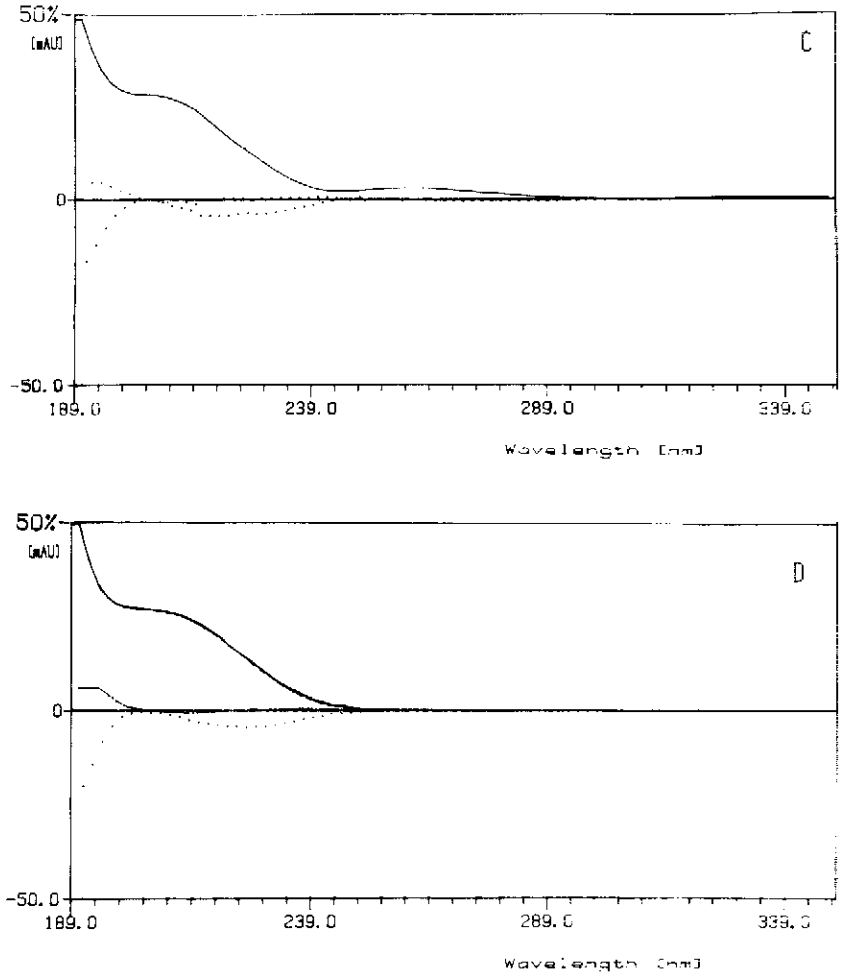

Fig. 3. (A) The 190-nm urinary HPLC carboxylic acid profile from a child with methylmalonic acidemia; the peak at 18.28 min is uric acid; (B) three-dimensional profile of the 6—12 min interval of the chromatogram; (C) 0th, 1st and 2nd derivative UV spectra of the 11.83-min peak; (D) 0th, 1st and 2nd derivative UV spectra of authentic methylmalonic acid.

normal (compare with Fig. 1). The three-dimensional plot (Fig. 3B) and the 0th, 1st and 2nd derivative UV spectra (Fig. 3C) of this peak indicate that it is methylmalonic acid. The 0th, 1st and 2nd derivative UV spectra of authentic methylmalonic acid are shown in Fig. 3D.

The 190-nm urinary carboxylic acid profile shown in Fig. 4A has an abnormally large peak at 8.36 min. Based upon RRT values, this peak could be orotic acid or 2-oxoglutaric acid. The three-dimensional plot of this peak is indicative of a 2-oxocarboxylic acid not orotic acid (compare Fig. 4B with Fig. 2B), Thus, this peak was identified as 2-oxoglutaric acid. GC—MS analysis of the TMS derivatives of the extracted urinary organic acids showed that 2-oxoglutaric acid was indeed elevated.

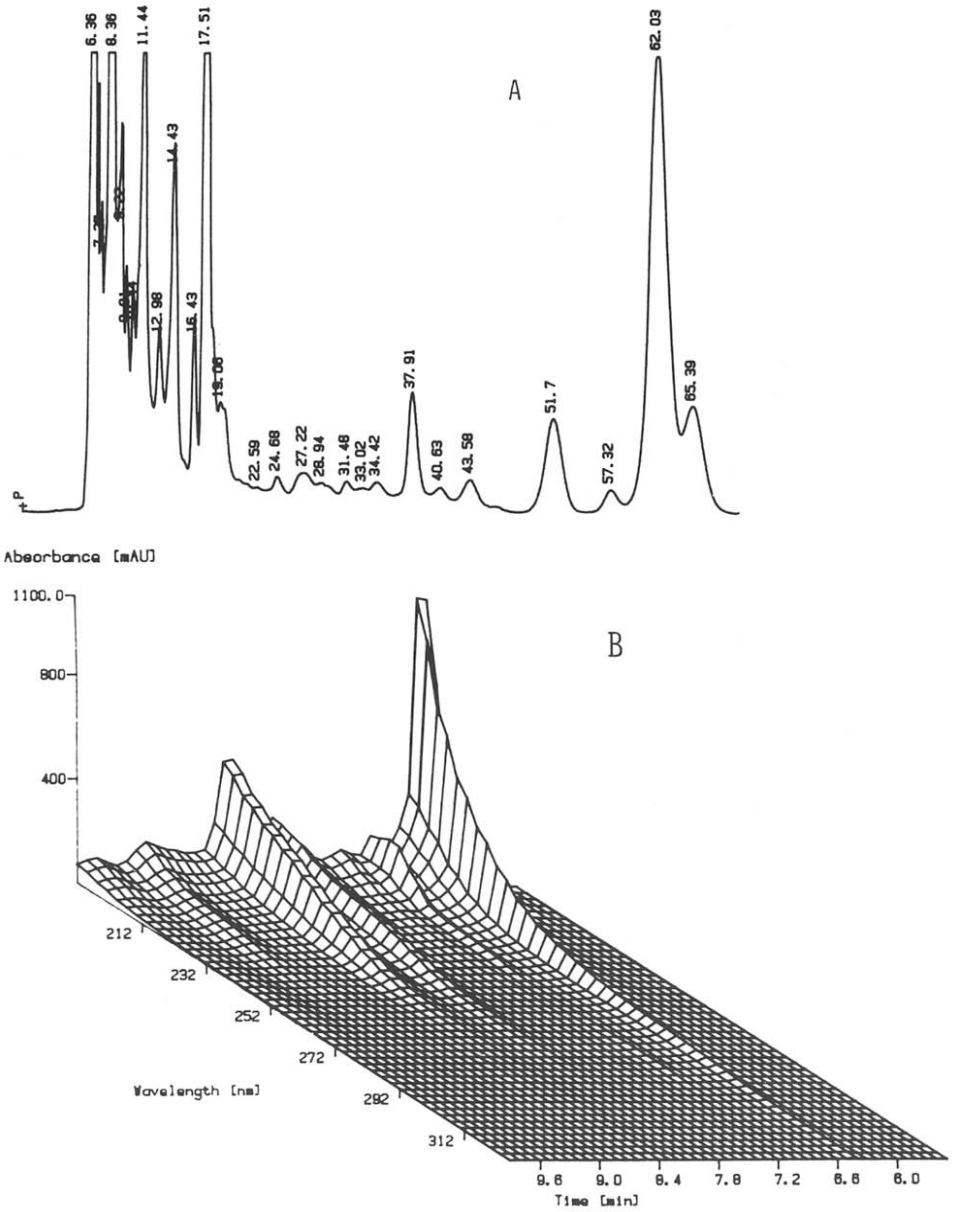

Fig. 4. (A) The 190-nm urinary carboxylic acid profile. The peak at 17.51 is uric acid while the peak at 62.03 min is hippuric acid and 4-hydroxyphenylacetic acid; (B) three-dimensional profile of the 5.5—10 min interval of the chromatogram. The peak at 8.36 min is 2-oxoglutaric acid.

As can be seen by comparing the HPLC carboxylic acid profiles in Figs. 1, 3A and 4A, there is some day-to-day and run-to-run variation in the elution volume of the compounds. Use of the photodiode array detector to produce the UV spectrum of each peak enables one to identify the internal standard (3-hydroxy-4-methoxybenzyl alcohol) and thus calculate the RRT of each peak.

Successful diagnosis and treatment of newborns with catastrophic inborn errors of metabolism requires that the time for analysis be kept to an absolute minimum. A delay in even one or two days may result in irreparable brain damage in the patient while the appropriate therapeutic avenue is sought. Because of the limited sample preparation and the brief (70—80 min) analysis time, we feel that the procedures described in this paper represent a significant advance in the ability to detect certain inborn errors of metabolism. By permitting one to distinguish various carboxylic acids via their UV spectra, it is possible to arrive at a diagnosis of some inborn errors with greater rapidity than was previously possible using GC—MS analysis of the TMS derivatives of the carboxylic acids. Although this procedure does not allow the same amount of information to be obtained as a complete analysis of the TMS-derivatized carboxylic acids via GC—MS, it represents an enhanced first step which can be performed while the more complete analysis is undertaken. In those instances in which information leading to a diagnosis is possible, the delay between diagnosis and institution of appropriate therapy is reduced accordingly.

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